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THE JOURNAL OF PARASITOLOGY

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SOME CONSIDERATIONS RELATIVE TO THE NATURE OF IMMUNITY IN *TRYPANOSOMA LEWISI* INFECTIONS

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It was recently demonstrated in our laboratory (Thillet and Chandler, 1957) that rats could be rendered completely refractory to *Trypanosoma lewisi* infections, even when given challenge doses of 20 million trypanosomes, by repeated injections of the metabolic products of the trypanosomes collected in a mixture of rat serum and saline over a period of 24 hours, after complete removal of the trypanosomes, still intact and alive. It was also demonstrated that the serum of rats immunized with metabolic products, like that of rats recovered from a natural infection, caused rapid agglutination of trypanosomes *in vitro*, whereas serum from rats in which immunization had been attempted by injections of dead, triturated trypanosomes had practically no agglutinating potency. And, finally, it was possible to remove most of the protective value of serum from recovered rats, including its inhibition of reproduction, by absorption with lyophilized metabolic products, but not by lyophilized washed trypanosome bodies.

These results obviously suggest that the resistance of rats to reinfection that follows recovery from a *T. lewisi* infection is due primarily to antibodies directed against metabolic products. The results of the absorption experiments indicate that these antibodies are identical with the reproduction-inhibiting antibody, or ablastin, observed by Taliaferro many years ago, although the inhibition of reproduction was not directly demonstrated in rats immunized by injections of metabolic products because of the rapid disappearance of the challenge trypanosomes—the same difficulty that Taliaferro (1932) had with some of his experiments with passive immunization. The conclusion seems warranted that these antibodies against metabolic products are also primarily, and possibly exclusively, responsible for the *in vitro* destruction of the trypanosomes at the first number crisis, and possibly, in part at least, for their final elimination from the host.

Earlier workers have disagreed on the variety of antibodies involved in the development of immunity to *T. lewisi*, and on the exact mechanism of that immunity. Francis (1903) considered only agglutination as a factor; he concluded that this "is a step towards dissolution and that it foretells the disappearance of the trypanosomes from the blood." Laveran and Mesnil (1901, 1912) and Manteufel (1909) clearly differentiated between an agglutinin which develops early in a primary infection, and an immobilizing (trypanocidal) antibody which appears only after repeated inoculation of trypanosomes, and requires the presence of complement.

Taliaferro and Taliaferro (1922) demonstrated what they interpreted as 2 types

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of resistance to *T. lewisi* infections in rats, first "an ever-increasing resistance which retards the rate of reproduction of the trypanosomes"; then, "just as the resistance against reproduction is reaching its climax, the host suddenly develops a resistance which destroys a majority of the organisms in the blood. The remaining parasites live in the blood for varying lengths of time (sometimes several months) until they are suddenly destroyed as the result of the final resistance which the host builds up against them."

In a subsequent paper, Taliaferro (1924) ascribed the inhibition of reproduction to a "reaction product" which he later (1932) recognized as an antibody (ablastin). On the basis of his own work and that of Coventry (1925, 1930), he postulated (1932) that 3 antibodies were concerned in the recovery of rats from a *T. lewisi* infection: (1) the reproduction-inhibiting antibody (ablastin) which first appears about the fifth day, increases rapidly in titer in the next few days, and then more gradually until about the 35th day, and then slowly decreases; (2) a trypanocidal antibody which accounts for the number crisis about the 10th day, when the majority of the non-reproducing trypanosomes disappear, and (3) a trypanocidal antibody associated with the final elimination of the parasites, to terminate the infection several weeks to several months later.

Augustine (1943) attributed recovery from reinfections entirely to trypanocidal antibody which sensitizes dividing cells and thus renders them vulnerable to phagocytosis, and which agglutinates adult parasites which are then mechanically removed from the circulation. He thought it possible that 2 antibodies were involved, an opsonin and an agglutinin, but preferred to regard the trypanocidal antibody as a single sensitizing agent. Finally Barnes (1951) recognized 3 distinct types of antibodies—ablastin, trpanolysin *and* agglutinin.

The demonstration that it is an antibody directed against metabolic products, presumably identical with ablastin, and not a trypanocidal antibody that is the cause of agglutination of *T. lewisi* accounts for much of the confusion that has existed in the past with regard to the immunological relations of this parasite, and suggests new interpretations of some of the results obtained by earlier workers. Briefly, the course of events seems to us to be as follows: antigenic metabolic products, which we may call ablastinogens, probably involving enzymes necessary for adequate nutrition of the parasites, begin stimulating ablastin formation on about the 5th day of an infection. Within a day or two later the ablastin, by neutralizing the metabolically necessary excretions that stimulated it, suppresses growth and reproduction. A day or two later, ablastin is present in sufficient quantity to produce a viscous antigen-antibody complex that causes the parasites to adhere to each other and thus agglutinate, without injuring them or destroying their motility; such parasites also adhere to platelets, leucocytes, etc. The adherence by the posterior ends to form rosettes may be due to excretion of the ablastinogen from that region, possibly as a result of activity of the kinetoplast, as von Prowazek (1905) suggested, or it may be that the motility of the organisms prevents the ablastinogen from adhering to other parts of the body long enough for the ablastin to combine with it in contact with the body. Laveran and Mesnil's demonstration that immobile parasites agglutinate in a helter-skelter manner give some support to this idea.

As soon as the parasites begin to agglutinate they are, as Augustine (1943) showed, mechanically filtered out in liver, spleen, etc., and rendered easy prey to

phagocytic action; this may be due, in part, to their impeded circulation, in part to their viscosity, and in part to the opsonic effect of the antigen-antibody complex adhering to them. This susceptibility to phagocytosis is probably sufficient to account for the first number crisis. As Coventry (1925) pointed out, this occurs at a time when the titer of ablastin is rapidly rising. Up to a few days prior to this number crisis, when their reproduction was inhibited, the parasites were increasing logarithmically, and there is no evidence of any appreciable number of trypanosomes having been destroyed to provide antigens for the stimulation of a trypanocidal antibody. When the parasites have become so reduced in number that opportunity for contact in the blood, and consequent agglutination, is greatly reduced, destruction by phagocytosis would probably decrease. Final elimination of the remaining trypanosomes may be due to gradual phagocytic elimination as the titer of ablastin increases, or to their destruction by an immobilizing trypanocidal antibody stimulated by the destruction of numerous trypanosomes at the first number crisis, or to a combination of both. That such an antibody is produced when a host has been exposed to adequate amounts of disintegrated trypanosome bodies was demonstrated by Laveran and Mesnil (1901), who regularly obtained an immobilizing antibody after 10 or more inoculations with trypanosomes, and by Taliaferro (1932), who obtained trypanocidal antibodies in 9 of 58 rats after a single hyper-immunization. It was also demonstrated by Taliaferro (1932) in rabbits repeatedly injected with trypanosomes.

Although ablastinogen is excreted by the parasites, it is evident that, as might be expected, the bodies of the parasites contain a small amount of this substance that has been elaborated but has not yet been excreted. Taliaferro (1932), for instance, showed that injection of rats with either 1 or 3 massive doses of triturated trypanosomes (5 ml of a heavy suspension) resulted in complete inhibition of reproduction if a challenge infection was given 7 to 9 days later, after there had been time for ablastin to be produced. Significantly, however, these injections failed to cause the death of trypanosomes that were intravenously injected even after 9 days, although the amount of trypanosome body substance used must have greatly exceeded the number of trypanosomes that could have been disintegrated prior to, or even after, a number crisis in a natural infection. Thillet and Chandler (1957) got normal infections when 1000 million triturated trypanosomes were used for immunization, but when 4000 million were used, they obtained infections with low peaks of trypanosomes and of relatively short duration. It seems evident that this amount of trypanosome body substance contained enough ablastinogen to initiate ablastin production so that reproduction was partially suppressed, and enabled the ablastinogen produced by the living challenge trypanosomes to stimulate a fully protective titer of ablastin in a shorter time.

Augustine (1943), who mistakenly attributed agglutination of *T. lewisi* to a trypanocidal antibody, observed no agglutination or destruction of trypanosomes within the peritoneal cavity of reinfected rats when large numbers were inoculated; the parasites rapidly escaped, uninjured, into the blood stream, where they were agglutinated and then removed by phagocytic action. From this, he concluded that the "trypanocidal" antibody is definitely located in the blood and has no effect upon the trypanosomes remaining in the peritoneal cavity. However, an alternative explanation is that the ablastinogen excreted by the very large number of the para-

sites in the peritoneal cavity would quickly neutralize the limited amount of ablastin present in it, or entering it from the general circulation. It seems quite possible that there would not be enough ablastin available in the body cavity to prevent reproduction from beginning even though there might be enough in the blood to agglutinate the parasites as they entered it. Thus it would be possible for dividing forms to appear in the blood over a period of several days, without any appreciable increase in numbers, as Augustine found to be the case. When Augustine injected actively multiplying trypanosomes directly into the heart, agglutination occurred almost at once, and the parasites were removed within 15 minutes.

In antibody-absorption experiments, Thillet and Chandler found that immune serum from recovered rats lost much of its protective power when absorbed with metabolic products, but not when absorbed with triturated trypanosomes. Taliaferro (1932), on the other hand, reported practically complete loss of curative and protective power of hyperimmune serum after absorption with large numbers (at least 1,000,000 per cmm) of living trypanosomes. These were left in contact with the serum for 6 hours on ice followed by 1 hour at 37° C. Taliaferro interpreted this as indicating absorption of antibody by the trypanosomes, and since he found that ablastin was *not* absorbed by the trypanosomes and did not kill them, since they resumed multiplication when transferred to a fresh rat, he concluded that this was clear indication of a separate trypanocidal antibody.

An alternative explanation is that the time during which this large number of living trypanosomes was left in contact with the serum was sufficient to allow them to excrete enough ablastinogens to combine with the ablastin that was present, and eliminate the curative and protective potency of the serum in this manner. Taliaferro observed that when absorption was incomplete, trypanosomes removed from the serum were "sensitized" and disappeared within a few minutes when removed and injected into a rat, whereas when absorption was complete, trypanosomes removed and injected into a rat did not all disappear. A possible explanation for this is that as long as the ablastin in the serum is not completely neutralized by ablastinogen produced by the parasites, it will cause the trypanosomes to agglutinate and thus to disappear by being rapidly filtered out of the blood stream when injected into rats, whereas in the presence of an excess of ablastinogen, or even of trypanosomes, there may not be enough ablastin to cause more than weak agglutination, with subsequent separation of the trypanosomes when injected into a rat.

In another experiment, Taliaferro observed no reduction in inhibition of reproduction, but some reduction in what he considered trypanocidal activity (based on disappearance of trypanosomes *in vivo*), when hyperimmune serum absorbed by living adult trypanosomes was injected into rats, but when the serum was absorbed twice, once with adult trypanosomes and subsequently with dividing trypanosomes, there was considerable reduction in inhibition of reproduction and very marked loss of "trypanocidal" power. In this experiment, it should be noted, the immune serum, when doubly absorbed, was exposed to living trypanosomes for a total of 2 hours. Also, in this experiment, the absorption apparently was done for an hour at 37° C *before* overnight absorption in the ice box; this could conceivably have resulted in greater excretion of ablastinogen by the parasites used for absorption.

In still another experiment, in which serum collected on the 21st or 22nd day after infection was absorbed by living trypanosomes, the potency of the serum in

inhibiting reproduction was unaffected. However, when the trypanosomes used for absorption were subsequently injected into rats, they were largely destroyed, in some cases disappearing from the blood for 2 days before they reappeared, rapidly dividing. This might well have been due to weak agglutination resulting from the large numbers of parasites used for absorption relative to the amount of ablastin present. Results of this kind might be expected, since it is now evident that it takes less ablastin to inhibit reproduction than to cause agglutination. It seems probable that these variable results in absorption experiments with living trypanosomes are related to quantitative differences in titer of ablastin in the serum and in numbers of trypanosomes used for absorption, and to experimental conditions relative to time and temperature of absorption.

Taliaferro (1938) experimented with the effects of passive transfer of "ablastic" and "trypanocidal" serum to normal and to splenectomized and "blockaded" rats a few minutes after they had been intravenously injected with large numbers of trypanosomes. The ablastic serum (taken from still infected rats 20 to 25 days after infection) was found to be equally effective in preventing reproduction in both normal and in splenectomized and blockaded rats for the first few days (usually 6) after which the parasites began to reproduce actively in the modified experimental rats, but not in the normal rats, undoubtedly due to the active production of ablastin in the latter but not in the former. When "trypanocidal" serum was transferred to these 2 types of rats, there was evidence of slight but definite impairment of its ability to cause disappearance of the parasites in the treated rats during the first few days. This, we believe, might well have been due to reduced phagocytosis of agglutinated parasites in the splenectomized and blockaded rats.

The exact nature of the ablastinogen is at present unknown. It is evidently an excreted metabolic product which enables the parasites to avail themselves of some constituent or constituents of the environment that is required for growth and reproduction. When this metabolic product is neutralized by ablastin, it ceases to function. It is an interesting hypothesis, at present entirely speculative, that the ablastinogen is an enzyme by means of which the parasites release hematin or protoporphyrin from hemoglobin for use in their iron-catalyzed respiration. The need for such compounds, or sources of them in hemoglobin, has been amply demonstrated by M. Lwoff (1933, 1939, 1951). The fact that rats in which trypanosome reproduction is uninhibited become anemic (Saul and Becker, 1949) could conceivably be due to continued enzymatic breakdown of hemoglobin. Moulder (1948) observed changes in glucose metabolism and a *rise* in R.Q. in non-reproducing compared with reproducing trypanosomes; however, although ablastin stopped reproduction, it does not follow that it was the cause of the subsequent change in metabolism observed in washed trypanosomes.

Deficiency of pantothenic acid has been found to increase parasite peaks and to prolong reproductive activity of *T. lewisi,* therefore presumably interfering with the production or activity of ablastin (Becker, Manresa and Johnson, 1943; and Becker, Taylor and Fuhrmeister, 1947). Similar effects were reported by Becker and Gallagher (1947), Saul and Becker (1949), and Barnes (1951) for administration of sodium salicylate and related substances.

Becker and Gallagher hypothesized that ablastin might be an oxidative enzyme for which pantothenic acid serves as a coenzyme, and that salicylate might usurp

the place of the pantothenic acid on the enzyme without being metabolically active, but Lysenko later (1951) reported experiments indicating that salicylate did *not* affect ablastin by entering into combination with it, but rather by interfering with its production. The most probable explanation for the anti-ablastic action of pantothenic acid is its demonstrated interference with the formation of antibodies (see Axelrod and Pruzansky, 1955). Salicylic acid probably also interferes with ablastin-production but, since Lysenko found no reduction of pantothenic acid in salicylate-treated rats, it is probably by a separate and independent mechanism, perhaps by an ACTH-like effect as suggested by Meyers and Lysenko (1953).

Barnes (1951) found that in rats treated with benzene the trypanosome population at the peak of the infection was larger, and reduction in numbers after the peak was more gradual, so that the length of the patent period was increased. Significantly, the time when the parasites ceased to reproduce was unchanged, and agglutination was not interfered with. Barnes attributed these changes to an effect of benzene on trypanolysin, but not on ablastin. However, benzene is well known to destroy leucocytes, producing a leucopenia, and may affect other phagocytic cells. Barnes herself observed a marked decrease in leucocytes, and particularly of mononuclear cells. Interference with phagocytosis seems to us to be an adequate explanation for Barnes' observations, just as it is for Taliaferro's (1938) observations on the effect of splenectomy and blockade. Since Barnes believed that salicylate interferes with ablastin and agglutinin, and benzene with trypanolysin, she expected administration of both substances to result in continuous pathogenic infections. However, since successful phagocytosis depends on prior agglutination of the parasites, we should expect little additional effect from the addition of an anti-phagocyte agent if agglutination has been prevented, and this is what Barnes found to be the case.

SUMMARY

The demonstration that ablastin is an antibody directed against metabolic products, that this is the cause of agglutination as well as inhibition of reproduction of *Trypanosoma lewisi*, and that immune serum from recovered rats loses most of its potency when absorbed with metabolic products, indicates that ablastin is primarily responsible for immunity of rats to reinfection, and is also the agent responsible for at least the first number crisis, since agglutination is a precursor of phagocytic destruction of the parasites.

Inhibition of reproduction, which is effected by a lower titer of ablastin than is agglutination, is presumably due to neutralization by antibodies of excreted metabolic products, probably enzymes, which enable the parasites to avail themselves of some constituent or constituents of the environment that is required for growth and reproduction. The agglutination appears to be due to ablastin-ablastinogen combination in contact with the parasite bodies.

Many experiments of earlier workers which seemed to indicate separate reproduction-inhibiting and trypanocidal antibodies seem to be adequately explained by quantitative relationships between ablastin and ablastinogen.

The modifications in the course of *T. lewisi* infections resulting from deficiency of pantothenic acid and from administration of salicylates, demonstrated by Becker and his colleagues, seem explicable on the basis of interference with antibody (ablastin)-production, whereas the effect of benzene demonstrated by Barnes, like

the effect of splenectomy and blockade demonstrated by Taliaferro, seem explicable on the basis of partial interference with phagocytosis.

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OBSERVATIONS ON *PLASMODIUM CIRCUMFLEXUM* KIKUTH AND *P. VAUGHANI* NOVY AND MACNEAL FROM EAST PAKISTAN

MARSHALL LAIRD* AND FAIYAZ A. LARI**

Hewitt (1949) published a synopsis of the avian plasmodia, giving brief details of 14 species widely accepted as valid. One of these, *Plasmodium oti* Wolfson, 1936, has since fallen as a synonym of *P. hexamerium* Huff, 1935 (Manwell, 1949), but the number has been restored to 14 by the confirmation of the validity of *P. fallax* Schwetz, 1930, by Huff *et al* (1950). Only four of the generally recognized species, *P. relictum* (Grassi and Feletti, 1891), *P. rouxi* Sergent *et al*, 1928, *P. polare* Manwell, 1934 and *P. gallinaceum* Brumpt, 1935, have been reported from the entire Indian subcontinent.

There are numerous presumptive records of *P. relictum* (= *P. praecox*) from Indian birds, including captive ones in zoological gardens in other countries (Wenyon, 1926). Das Gupta and Siddons (1941) and Singh *et al* (1952) have given adequate accounts of this parasite from natural hosts in India itself. Ray and Bhatnagar (1953) found *P. rouxi* in the blood of a partridge from Delhi, Ray *et al* (1953) recorded *P. polare* from the same host and locality, and various workers (Mohan, 1955) have described experimental studies with *P. gallinaceum* derived from Ceylonese and Indian fowls.

Plasmodium heroni Basu, 1938, described from a heron at Calcutta but listed as a species of questionable status by Hewitt (1949), is probably a synonym of *P. circumflexum* or *P. fallax* (see page 142). Seven other plasmodia (overlooked by Hewitt) have been recorded from the subcontinent: *Plasmodium moruony* (de Mello and de Sá, 1916) (Bhatia, 1938), *P. chloropsidis* de Mello, 1936, *P. gallinulae* de Mello, 1936, *P. herodialis* de Mello, 1936, *P. centropi* de Mello, 1936, *P. ploceii* Chakravarty and Kar, 1945 and *P. pericrocoti* Chakravarty and Kar, 1945. De Mello's "species" were founded on the indefensible contention that "every bird generally has its own plasmodid and sometimes more than one" (de Mello, 1936a), and available data concerning all 7 parasites are too limited either to establish their validity or to permit their transfer to accepted species.

Our knowledge of the occurrence of bird malaria parasites in India itself is thus very far from complete, many of the records being of no more than generic significance. There are no published records whatsoever for Pakistan, and careful and comparative accounts for the whole subcontinent are much to be desired to fill in a major gap in the distribution map of avian plasmodia. The purposes of this paper are to summarize the results of a haematozoan survey in East Pakistan, to present morphological accounts of 2 widely distributed species occurring there, and to discuss relevant problems of nomenclature.

SURVEY DATA

Avian blood smears were collected at the Laboratory of the Malaria Institute of Pakistan, Dacca, East Pakistan, from February, 1953, to January, 1954. The birds,

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obtained through dealers, originated from the district of Sylhet bordering the Indian Province of Assam.

Thin smears of peripheral blood were made from each bird, and stained with Giemsa. Subinoculations into laboratory birds were undertaken wherever possible. Some of the films were 3 years old when this account was prepared, but it was found that slight fading of the stain could be compensated for by examination under oil immersion with phase contrast illumination. The technique proved particularly valuable in elucidating the shape of the merozoites of *P. circumflexum*. These are elongate structures, but the delicate cytoplasmic extremities lose their stain quite rapidly in Giemsa preparations and the bright field picture becomes misleading, the merozoites appearing oval. The survey findings are summarized in Table I.

TABLE I. *Analysis of blood smear records.*

Previous Indian Records[1]							HOSTS[2]		Present Surveys[3]			
Trypanosoma	*Haemoproteus*	*Leucocytozoon*	*Plasmodium*	*Microfilaria*	*Atoxoplasma*	Other	Systematic position	Common name	Number examined	Number positive	*Haemoproteus*	*Plasmodium circumflexum* *Plasmodium vaughani*/ *P. circumflexum* *P. vaughani*
							ANATIFORMES					
							Anatidae	Domestic Duck	2			
							FALCONIFORMES					
							Accipitridae					
							Gyps bengalensis (Gmelin)	Indian White-backed Vulture	1	1	1	
							GALLIFORMES					
			LAB			X	Gallidae	Domestic Fowl	2			
							COLUMBIFORMES					
							Columbidae					
X	X				X		*Columba livia* Gmelin	Rock Pigeon	4	1	1	
							Streptopelia chinensis suratensis (Gmelin)	Indian Spotted Dove	3	1	1	LAB
							CUCULIFORMES					
							Cuculidae					
							Eudynamys scolopacea (L.)	Koel	1			
							STRIGIFORMES					
							Strigidae					
	X	X					*Athene brama indica* (Franklin)	Northern Spotted Owlet	1			
							CORACIIFORMES					
							Capitonidae					
	X						*Megalaima haemacephala indica* (Latham)	Burmese Crimson-breasted Barbet	3			
	X			X			*Magalaima zeylanica caniceps* (Franklin)	Northen Green Barbet	2			
							Alcedinidae					
	X						*Halcyon smyrnensis fusca* (Bodd)	Indian White-breasted Kingfisher	1	1	1	
							Meropidae					
	X						*Merops orientalis orientalis* Latham	Indian Green Bee-eater	1			
							Picidae					
	X	X					*Dinopium benghalense benghalense* (L.)	Northern Golden-backed Woodpecker	1			
							PASSERIFORMES					
							Corvidae					
X	X		X			X	*Corvus splendens splendens* Vieillot	Common Indian House Crow	2			
							Dicruridae					
	X			X			*Dicrurus macrocercus peninsularis* Ticehurst	Indian Black Drongo	2			
							Laniidae					
							Lanius sp.	Shrike	1			
							Oriolidae					
		X		X			*Oriolus xanthornus xanthornus* (L.)	Indian Black-headed Oriole	2			
							Ploceidae					
X	X		X		X		*Passer domesticus indicus* Jardine & Salby	Indian House Sparrow	94			
X	X		X				*Lonchura malabarica* (L.)	White-throated Munia	3			

TABLE I. *Analysis of blood smear records.* (*Continued*)

Previous Indian Records[1]							HOSTS[2]		Present Surveys[3]					
Trypanosoma	*Haemoproteus*	*Leucocytozoon*	*Plasmodium*	*Microfilaria*	*Atoxoplasma*	Other	Systematic position	Common name	Number examined	Number positive	*Haemoproteus*	*Plasmodium circumflexum*	*Plasmodium vaughani/ P. circumflexum*	*P. vaughani*
							Lonchura punctulata (L.)	Spotted Munia	3					
							Pycnonotidae							
X		X		X			*Pycnonotus cafer bengalensis* Blyth	Bengal Red-vented Bulbul	2					
	X		X				*Pycnonotus jocosus emerius* (L.)	Bengal Red-whiskered Bulbul	2					
							Sturnidae							
	X						*Acridotheres fuscus fuscus* (Wagler)	Indian Jungle Mynah	4			LAB		
				X			*Acridotheres tristis tristis* (L.)	Common Indian Mynah	76	5		3	1	1
							Sturnus contra contra (L.)	Assam Pied Mynah	3					
	X						*Sturnus malabaricus malabaricus* (Gmelin)	Indian Grey-headed Mynah	42	13	1	5	7	
X							*Sturnus pagodarum* (Gmelin)	Black-headed Mynah	1					
							Turdidae							
X	X			X		?	*Copsychus saularis* (L.)	Magpie Robin	3					
								Totals	262	22	5	8	8	1

[1] The entry for *Corvus splendens* in the "Other" column refers to a species of *Babesia* dealt with elsewhere (Laird and Lari, 1957), and that for the domestic fowl concerns the widespread *Aegyptianella pullorum* Carpano and another babesioid recorded by Abdussalam (1954). The remaining records are extracted from the following publications.—Wenyon (1926), de Mello (1936a, 1936b, 1936c), Das Gupta and Siddons (1941), Chakravarty and Kar (1945), Zargar (1945), Singh *et al* (1951) and David and Nair (1955).

[2] The common names of the birds examined are after Stuart Baker (1932–35), but we have followed modern ornithological usage (c.f. Biswas, 1952) in the matter of the generic status of many species.—

Generic name used herein	Supplanted generic name	Species
Acridotheres	(*Aethiopsar*)	*fuscus*
Dinopium	(*Brachypternus*)	*benghalense*
Gyps	(*Pseudogyps*)	*bengalensis*
Lonchura	(*Uroloncha*)	*malabarica*
Lonchura	(*Uroloncha*)	*punctulata*
Megalaima	(*Thereiceryx*)	*zeylanica*
Megalaima	(*Xantholaema*)	*haemacephala*
Pycnonotus	(*Molpastes*)	*cafer*
Pycnonotus	(*Otocompsa*)	*jocosus*
Sturnus	(*Sturnia*)	*malabaricus*
Sturnus	(*Sturnopastor*)	*contra*
Sturnus	(*Temenuchus*)	*pagodarum*

[3] *Streptopelia chinensis suratensis* and *Acridotheres fuscus fuscus*, neither of which were previously known to harbour *Plasmodium*, were experimentally infected with *P. circumflexum* in the laboratory at Dacca.

As is evident from Table I, 262 birds of 27 species were dealt with. Natural haematozoan infections were found in only 22 birds of 6 species, 2 of which (*Gyps bengalensis* and *Streptopelia chinensis suratensis*) are new hosts for blood parasites. A further species, *Acridotheres fuscus fuscus*, proved highly susceptible to *Plasmodium circumflexum* in the laboratory.

Fifteen of the other 20 species of birds were already known to support haematozoa of one description or another in other parts of the Indian subcontinent. A further one, the domestic duck, is a common host notably for *Leucocytozoon* elsewhere, and the remaining 4 belong to genera (*Eudynamys, Lanius, Sturnus,* and *Lonchura*) of which other species harbour blood parasites.

Since haematozoa are known from 23 of the species dealt with and from other species of the genera to which the remaining 4 birds belong, it may be wondered why the survey disclosed only 6 hosts for blood parasites. This is less strange, however, in the light of the fact that relatively small numbers of most of the species

were handled. Thus the number of examples of a given species exceeded 4 in only 3 cases, those of *Passer domesticus indicus* (94), *Acridotheres tristis tristis* (76) and *Sturnus malabaricus malabaricus* (42); and only 5 (7%) of the second of these birds and 13 (31%) of the third proved to be parasitized. It is surprising that the examination of as many as 94 Indian house sparrows should yield completely negative results. *Trypanosoma, Haemoproteus, Plasmodium* and *Atoxoplasma* have all been recorded from this host elsewhere in the subcontinent, *P. relictum* infections in particular being common and widespread. The latter parasite was found in 235 (27.5%) of 869 examples of this host in Delhi by Singh *et al* (1951), and it is also common in Karachi house sparrows (F.A.L.).

A second point for comment is the discovery of so limited a range of haematozoans in a random survey of 27 species of birds including several obviously "good" hosts, *Haemoproteus* and *Plasmodium* being the only genera encountered. There are Indian records of *Haemoproteus* from 15 of the species in question, of *Trypanosoma* from 7, of *Microfilaria* (*sens. lat.*) from 6, of *Plasmodium* from 5 (possibly 6), of *Leucocytozoon* from 4, of *Atoxoplasma* from 2 and of a babesioid from 2. The absence of *Trypanosoma* may of course be apparent rather than real, avian infections very frequently being cryptic (David and Nair, 1955), but the non-discovery of other haematozoans, especially *Leucocytozoon* and *Microfilaria*, is unusual. Further investigations of these matters are required, with particular attention to the occurrence and distribution of arthropod vectors.

The 5 *Haemoproteus* infections recorded herein are being dealt with separately. Neither of the natural hosts for *Plasmodium, Acridotheres t. tristis* and *Sturnus m. malabaricus*, were previously known to harbor parasites of this genus. The former bird, from which the only haematozoan recorded in India is *Microfilaria acridotheris* de Mello (1936c), harbours *Microfilaria mynah* Laird (1951) in Fiji and *Trypanosoma, Haemoproteus* and *Leucocytozoon* in Mauritius (Maya and David, 1912) introductions into both localities having been made from India. There is only one previous record of a blood parasite from *Sturnus m. malabaricus*, that of *Haemoproteus sturni* de Mello (1936a) from Ponda, India.

The *P. circumflexum* and *P. vaughani* infections were recorded between September 14, 1953 and January 2, 1954, but as nearly all examples of the 2 natural hosts were collected in the same period, the figures are of no assistance in assessing seasonal incidence.

P. circumflexum derived from captured *Sturnus m. malabaricus* was successfully subinoculated into *Acridotheres f. fuscus, Acridotheres t. tristis* and *Streptopelia chinensis suratensis*. While one example of the last-named species proved highly susceptible another was refractory to infection, as were sparrows, pigeons, ducks and fowls. Attempts to transfer *P. vaughani* to sparrows were also unsuccessful.

Our morphological account of *P. circumflexum* has been prepared from slides from 2 light natural infections in *Sturnus m. malabaricus* and from 2 heavy laboratory infections in the same host and in *Acridotheres t. tristis;* that of *P. vaughani* is from a single heavy natural infection in *Sturnus m. malabaricus*.

MORPHOLOGICAL ACCOUNT

(All illustrations prepared with the aid of a Zeiss-Winkel drawing apparatus at a magnification of 2,850×, all averages computed from the measurement of 100 successive examples, and all measurements in microns.)

Plasmodium circumflexum Kikuth, 1931
(Plate I, Figures 1–22)

Trophozoites: As many as 4 (Fig. 1) or 5 (Fig. 2) merozoites invading a single erythroblast. Signet ring stages occurring in both polychromatophil erythroblasts and normoblasts (Fig. 3), their location usually being polar as is that of amoeboid trophozoites in young erythrocytes (Fig. 4).

Schizonts: Presegmenters having 5 or 6 (Figs. 6, 12) nuclei common, their length along the midline averaging 14.7 (range, 10.2–16.8) as compared with 13.6 (range, 12.2–14.9) for the host erythrocytes. The following nuclear division most commonly synchronous and in the lateral plane (Fig. 7). The brownish-black to golden pigment granules rounded, oval or bacilloid, characteristically massed together at or towards one end of the parasite. Schizonts having 18 or more nuclei rare, 26 being the highest number recorded (Fig. 10).

Merozoites: A count for 100 successive segmenters from a slide in which these forms were dominant yielded the following percentages:

10	merozoites—	3%
12	"	—27%
14	"	—40%
16	"	—21%
18	"	— 5%
20	"	— 2%
22	"	— 1%
24	"	— 1%

Intraerythrocytic merozoites pointed at one end and rounded at the other, (Figs. 14, 15, etc.), although this is not always evident through fading (page 141) and because some at least of them are oriented in an end-on position. The characteristic shape more obvious in those free in the plasma (Fig. 17a). Usually 3 or 4 large grains of pigment, often clumped together (Figs. 15, 17, 18), sometimes a single spherical pigment mass (Fig. 16).

Gametocytes: Growing gametocytes partly encircling the host cell nucles (Fig. 19), mature ones surrounding this structure completely (Figs. 20–22).

Parasite level and periodicity: Up to 570 parasites per 10,000 erythrocytes at the peak of an infection. A high degree of synchronism, schizonts (75% of them having from 4 to 6 nuclei) accounting for 98% of the total parasite burden in one instance, and an overwhelming dominance of mature segmenters and of polychromatophil erythroblasts recently invaded by merozoites being noted in another slide.

Multiple infections: Up to 3 presegmenters per host cell. Multiple invasion, usually ending in the filling of all the available space within the host cell, is disclosed by the presence of more than one pigment clump (Fig. 13).

Effects of the parasite on the host cell: Both schizonts (Figs. 6–10) and gametocytes, especially those that do not completely surround the host cell nucleus (Fig. 19), may push the latter aside. The examination of 100 successive erythrocytes containing elongate presegmenters revealed discernible (but often very slight) displacement in 77 instances. Slight lengthwise stretching of the host cell occurs commonly.

One hundred *Acridotheres* erythrocytes containing elongate presegmenters measured 12.2–14.9 (av., 13.6) by 5.6–7.2 (av., 6.0) whereas 100 uninfected ones (those chosen each being adjacent to one of the parasitized cells concerned, thus eliminating the possibility of error due to differential distortion in various parts of the smear) measured 11.9–14.9 (av., 13.2) by 6.1–7.5 (av., 6.6).

Systematic position: Only 2 of the 14 widely accepted bird malaria parasites, *P. circumflexum* and *P. lophurae* Coggeshall, 1938, have gametocytes which encircle the host cell nucleus. The usual criterion by means of which these 2 species are separated is that the first one forms more merozoites than the second, 13–30 (av. 19.8 ± 5) (Manwell and Wolfson, 1934) as compared with 9–19 (av. 14) (Hewitt, 1942). Recently however, Paraense (1952) has described a Brazilian strain of *P. circumflexum* which forms 6–22 (av. 13.5 ± 2.44) merozoites. Another aid to identification is afforded by the relatively high degree of synchronism displayed by *P. circumflexum* (Wolfson, 1936). This contrasts sharply with the low degree of synchronism of *P. lophurae,* in which all growth stages are always in evidence throughout an infection (Hewitt, 1942). Furthermore, merozoites of the latter

species do not invade erythroblasts (Coggeshall, 1938; Hewitt, 1940) but *P. circumflexum* merozoites always do so (Hegner and Eskridge, 1938).

The presence of occasional presegmenters having more than 19 (up to 26) nuclei, the high degree of synchronism, and the characteristic occurrence of invasive merozoites and the youngest ring form trophozoites in polychromatophil erythroblasts, all serve to distinguish the parasite under discussion from *P. lophurae*. Since Coggeshall (1938) described *P. lophurae* from a Borneo Fireback Pheasant, *Lophura igniti igniti*, in the New York Zoological Park (the strain has subsequently been maintained in chickens and other laboratory hosts in the U.S.A.), there has been but one field record of this species, that of Ogaki (1949) from a Malayan dove, *Geopelia striata*. Ogaki failed to supply morphological data to support his identification, and as he also found *P. circumflexum* in several hosts in the same locality his record must await confirmation.

It remains to establish whether or not the Pakistan parasite is referable to *P. circumflexum*. There is agreement in that the youngest trophozoites are to be found in erythroblasts, and the trophozoites are less frequently lateral than polar or subpolar in location (Fallis, 1945), as is the case with the present parasite. According to the data and illustrations of Kikuth (1931), Giovannola (1934) and Paraense (1952) the older schizonts of *P. circumflexum* are of elongate outline and lie alongside the host cell nucleus, their pigment being clumped, usually towards one extremity. Paraense made particular reference to this feature, stating "it is so characteristic as to indicate a double infection when two clusters of pigment appear in a single erythrocyte." The color and disposition of the pigment as described by Kikuth and Paraense hold good for the present species. Multiple invasion of host cells is common (Savage and McTavish, 1951; Paraense, 1952). Manwell and Goldstein (1939) were the first to draw attention to the coccidia-like appearance of *P. circumflexum* merozoites. Fallis (1945) saw rounded, ovate, lanceolate and elongate merozoites in almost equal numbers, while Paraense (1952) figured his as pointed at one end and broadly rounded at the other. The parasite under consideration agrees with *P. circumflexum* in all these features, and has gametocytes exactly comparable with those of this species and of *P. lophurae* as figured by various authors. Fallis (1946) working with strains of *P. circumflexum* and *P. lophurae*, found it impossible to distinguish the gametocytes of these 2 parasites from one another. The occurrence of some schizonts having from 20 to 26 nuclei, separating the Pakistan *Plasmodium* from *P. lophurae* but being in agreement with *P. circumflexum*, has already been referred to. Hewitt (1940, 1949) asserted that neither mature gametocytes nor asexual forms of *P. circumflexum* displace the host cell nucleus. Nevertheless, Kikuth (1931, Fig. 6) plainly illustrated appreciable lateral displacement and slight tilting of a host cell nucleus partly surrounded by a macrogametocyte; and nuclear displacement and host cell elongation and hypertrophy are all apparent in the photomicrographs of Giovannola (1934). Savage and McTavish (1951) observed that 29 of 100 schizonts of *P. circumflexum*, and 22 of 100 gametocytes, appreciably displaced the host cell nucleus, and Paraense (1952) found that 26.6% of the schizonts and 45.8% of the gametocytes of his strain of this species markedly did so, a further 24.4% of the gametocytes causing slight displacement. As to host specificity, *P. circumflexum* was first described from a thrush (the Fieldfare, *Turdus pilaris*) in Germany (Kikuth, 1931), and has since been reported from various other members of the family Turdidae including *T. iliaceus* in Italy (Giovannola, 1934), *T. migratorius* in the U.S.A. (Manwell and Herman,

1935) and *Copsychus saularis musicus* in the Federation of Malaya (Ogaki, 1949). The species is now known from various other passerine birds, one paper alone including records from representatives of 6 different families in the U.S.A. (Love *et al,* 1953). Non-passerine birds in which its occurrence has been established include the Canadian Ruffed Grouse, *Bonasa umbellus* (Galliformes) (Fallis, 1945, 1946) and Eider Duck, *Arctonetta fischeri,* (Anatiformes) (Savage and McTavish, 1951).

It is thus evident from the literature that *P. circumflexum* has more effect upon the host cell than is commonly assumed, and that its geographical distribution and host range are both so wide that neither the lack of previous reports from the Indian subcontinent nor from birds of the starling family (Sturnidae) can be regarded as bearing upon our problem of identification. The Pakistan parasite differs from German and some North American *P. circumflexum* in but 2 respects, the average and the slightly lower threshold of the merozoite number. However, a recently described Brazilian strain of *P. circumflexum* has an even lower merozoite threshold than ours (Paraense, 1952) and Fallis (1945) found no more than 22 and usually fewer than 20 merozoites in his strain from Canadian grouse. It is contended that the differences in the threshold and average of the merozoite number are too slight to stand as the only justification for the description of a new species, and that they are best regarded as pointing to a strain within a species already known to vary considerably in these respects. The strain in question appears close to that described by Paraense (1952) [from *Gnorimopsar c. chopi* (Icteridae), a Brazilian passerine bird] with 6–22 (av. 13.5 ± 2.44) merozoites.

While the schizonts and merozoites of *P. fallax* have much in common with those of *P. circumflexum,* the former species has elongate gametocytes closely resembling those of *Haemoproteus* (Schwetz, 1930; Huff *et al,* 1950), and it does not exhibit synchronism (Huff *et al,* 1950).

P. heroni Basu, 1938, has crescentic gametocytes. Basu failed to state whether mature gametocytes of his species ever completely surround the host cell nucleus, and he made no mention of any preference for the invasion of erythroblasts or reticulocytes. He did not succeed in transmitting the parasite to canaries by blood inoculation, this being regarded by Giovannola (1939) as affording the only grounds justifying its separation from *P. circumflexum.* There is otherwise morphological agreement between the blood stages of the latter species and *P. heroni.* Schizonts, with their elongate form and clumped pigment, are just the same in both cases. The average merozoite number for *P. heroni* (14) is identical with that of the present strain of *P. circumflexum* and the range in merozoite number is even greater (6–26). However, it cannot yet be stated with certainty whether *P. heroni* is a valid species or, more probably, a synonym of *P. circumflexum* or *P. fallax.*

In the light of the above discussion, our parasite is identified as *P. circumflexum.*

If it should prove that the gametocytes of *P. heroni* completely surround the host cell nucleus when mature, and that merozoites of this parasite invade erythroblasts rather than erythrocytes, the name must fall as a synonym of *P. circumflexum.* In all probability Basu's *Plasmodium* is referable to the strain of the latter species described herein, for there is not only close morphological agreement but also the type locality of *P. heroni,* Calcutta, is in East Bengal not far to the South-east of Sylhet whence our material was derived.

Plasmodium vaughani Novy and MacNeal, 1904
(Plate II, Figures 23–48)

Trophozoites: Merozoites invading erythroblasts (Figs. 23, 24), ring stages occurring in both polychromatophil elements and normoblasts (Figs. 25, 26). One or 2 small grains of pigment always present, and a refringent granule sometimes apparent (Fig. 28).
Schizonts: Four (Figs. 29, 30), 6 (Figs. 31–35, 40) or 8 (Figs. 36–38) nuclei. Up to 3 (Figs. 31, 32) pigment granules present, but characteristically 2, one of them large and refractile and the other small and dot-like (Figs. 36, 37, 40, etc) or bacilloid (Figs. 33, 41).
Merozoites: Chromatin staining intensely peripherally, the central zone virtually colorless. Individual merozoites often appearing wholly composed of chromatin, others surrounded by a clear halo perhaps indicative of cytoplasm. Counts yielded the following merozoite numbers.—

4	merozoites—	21%
6	"	—60%
8	"	—19%

Gametocytes: Elongate, often of irregular outline (Figs. 44, 46, 48), both macrogametocytes and microgametocytes ranging up to 12.6 in length.
Parasite level and periodicity: Up to 3,000 parasites counted per 10,000 red cells. All developmental stages present together in abundance, indicating a low degree of synchronism.
Multiple infections: Common, a count from 100 successive parasitized erythrocytes revealing 58 single, 38 double and four triple infections.

Effects of the parasite on the host cell: Slight lengthwise stretching of the parasitized erythrocyte characteristic, as is indicated by the following figures.—

Measurements of 100 normal erythroyctes of *Sturnus m. malabaricus*	11.1–14.0 × 4.9–5.9 (av., 12.8 × 5.4).
Measurements of 100 erythrocytes each containing 1 parasite	11.5–14.4 × 4.7–5.5 (av., 13.1 × 5.1).
Measurements of 100 erythrocytes each containing 2 parasites	12.0–15.1 × 4.7–5.4 (av., 13.5 × 5.0).

Systematic position: This *Plasmodium* bears comparison with 5 of the species recognized by Hewitt (1949), *P. vaughani* [synonyms: *P. tenue* (Laveran and Marullaz) and *P. tumbayaensis* (Mazza and Fiora)], *P. rouxi, P. nucleophilum* Manwell, 1935, *P. hexamerium* (synonym: *P. oti* Wolfson) and *P. juxtanucleare* Versiani and Gomes, 1941; and with a species of doubtful standing, *P. huffi* Muñiz *et al,* 1951.

There are close affinities between these species. They agree in having elongate gametocytes, in forming few merozoites per segmenter (typically 4–8), and in their low degree of synchronism; and none of them cause more than slight displacement of the host cell nucleus.

P. rouxi stands apart from the others in that the number of merozoites per segmenter is invariably 4 (Sergent *et al,* 1928). Most of the *P. rouxi* records are from North African passerine birds, but recent studies have established that the species occurs in pheasants in Iran (Rousselot, 1947) and India (Ray and Bhatnagar, 1953). *P. juxtanucleare,* described from the domestic fowl in Brazil and since recorded only from Mexico, may form as few as 3 merozoites per segmenter (range, 3–5); it differs further from the species under consideration in exhibiting round as well as elongate gametocytes (authors, in Hewitt, 1949). *P. nucleophilum* is known only from the U.S.A. It infects the Catbird, *Dumatella carolinensis* (Mimidae), and is transmissible to canaries. Manwell (1935a) at first regarded it as a variety of *P. vaughani,* but soon afterwards (1935b) described it as new, the specific name calling attention to the fact that both schizonts and gametocytes are nearly always closely applied to the host cell nucleus. Mature schizonts of *P. nucleophilum* tend to take up a polar position, and this, together with the intimate association with the host cell nucleus, holds for the species under consideration (Figs. 29–38).

Manwell (1935b) gave the number of merozoites as 4 to 9 (av. 6–7), but it was subsequently discovered that from 3 to 10 may be formed (av. 6.09 ± 0.29) (Manwell and Voter, 1939). This, together with the rarity of segmenting forms and the fact that the degree of parasitemia is much lower than in the case of *P. vaughani* (Manwell, 1935b; Manwell and Voter, 1939), serves to distinguish the two parasite from one another and eliminates *P. nucleophilum* from further comparisons with the present species. The erythrocytic stages of *P. huffi*, Muñiz *et al* are closely allied to those of *P. nucleophilum* as indicated by Manwell (Huff, 1953), and Huff (1956) has recently found such agreement between the exoerythrocytic stages of the two species as to suggest that they are conspecific.

P. hexamerium is known from many species of passerine birds in the U.S.A. (ref. Manwell, 1949, and Love *et al*, 1953, for hosts), the only non-American record being that of Levitanskaia and Lysenko (1952) from a finch in Russia. According to the original description (Huff, 1935) the chief feature separating this species from *P. vaughani* is the regular formation of 6 merozoites (never 4, seldom 8). However, Manwell (1949) found that although the usual number of merozoites per segmenter is 6 the range is from 4 to 8; and Khabir and Manwell (1955) reporting on a laboratory strain mantained in ducks for 8 years, stated that the average merozoite brood per segmenter had increased to 7.68 ± 0.12. Neither Huff (1935) nor Manwell (1949) mentioned the occurrence of refractile granules in *P. hexamerium*, but such granules, which are common in *P. vaughani* and less so in *P. nucleophilum*, were described by Wolfson (1936) for her *P. oti*, a parasite relegated to the position of a synonym of *P. hexamerium* by Manwell (1949).

Our Pakistan species may form fan-shaped schizonts like that figured in Plate I, Figure 9 of the original description of *P. hexamerium* (cf. Plate II, Figures 31, 32 herein), its schizonts are commonly located towards 1 pole of the host cell (as is the case with *P. hexamerium* according to Manwell, 1949), the pigment is clumped in schizonts and some gametocytes but is scattered in other gametocytes, and the merozoite index agrees with that of the typical strain of the latter species. Apart from possible physiological differences that will be referred to in due course, the two organisms appear identical in all respects but one. Huff (1935) stated that all stages of *P. hexamerium* occur in mature erythrocytes, and although he was dealing with relatively low parasitemias in which the occurrence of young individuals in immature red cells could easily have been overlooked, Khabir and Manwell (1955) found that even in cases where there is a parasitemia of 50% or more the species exhibits a marked preference for mature erythrocytes over reticulocytes. Trophozoites of the small parasite of *Sturnus m. malabaricus*, on the other hand, frequently invade polychromatophil erythroblasts as well as reticulocytes and erythrocytes.

The remaining species, *P. vaughani* itself, must now be discussed. Unlike *P. nucleophilum* and *P. hexamerium*, this parasite is well known outside North America. Besides parasitizing robins (*Turdus migratorius*) and starlings (*Sturnus vulgaris*) in the U.S.A. (Manwell, 1935a; Hegner and Wolfson, 1938) it has been reported from a wide variety of passerine birds in South America (Mazza and Fiora, 1930; Renjifo *et al*, 1952), ? Japan (Uegaki, 1930; and from a Japanese *Leiothrix* in France by Laveran and Marullaz, 1914), Formosa (Ogawa and Uegaki, 1927), Hawaii (Fisher and Baldwin, 1947), the New Hebrides (Laird, 1953), Kenya (Garnham, 1950) and Europe (Giovannola, 1934). The *Plasmodium* under consideration agrees with *P. vaughani* as redescribed by Manwell (1935a) and subsequent authors except in that 6 merozoites are formed more frequently than 4.

Most of the investigators who have dealt with the latter species have recorded a range of from 4 to 8 merozoites in mature segmenters, although Laveran and Marullaz (1914) and Garnham (1950) did not record more than 6 merozoites. Mazza and Fiora (1930), Manwell (1935a), Garnham (1950) and Laird (1953) dealt with strains in which a majority of segmenters exhibit 4 nuclei. Ogawa and Uegaki (1927) described the division of the chromatin into 2 masses, then into 4 or 6 and finally into 8, the inference being that 8 is the normal end number; and Giovannola (1934) stated that the number of merozoites formed by an Italian strain (= *P. tenue*) never exceeded 8 *but was more frequently 8 than 4* ("più spesse 8 che 4"). In other respects, notably in the occurrence of amoeboid trophozoites, the number of pigment granules in asexual stages, the presence of refractile granules, and the apparent absence of cytoplasm from segmenting stages and merozoites, morphological agreement is complete. While it is often stated that *P. vaughani* does not displace the host cell nucleus (*cf.* Hewitt, 1949), Manwell (1935a) and Laird (1953) observed that occasional displacement takes place, and Ogawa and Uegaki (1927) asserted that the nucleus is often slightly displaced. Relatively slight effects upon the host cell probably vary with the strain involved. Of more significance is the fact that *P. vaughani* exhibits a tendency to invade immature red cells (Hewitt, 1949), trophozoites occurring not only within reticulocytes but in basophil and polychromatophil erythroblasts as well (Laird, 1953). *P. nucleophilum* and the probably cospecific *P. huffi*, invade polychromatophil erythroblasts, the former according to Manwell and Voter (1939) and the latter according to Muñiz *et al* (1951) and Huff (1953). Reasons why the Pakistan parasite cannot be referred to these, nor to *P. rouxi* nor *P. juxtanucleare*, have already been given. It is contended that there would be no justification for describing the species as new in view of its close morphological agreement with both *P. vaughani* and *P. hexamerium*. Although the average merozoite number agrees with the latter species, this number varies in both parasites and it is thus inadmissible to base an identification upon merozoite number alone. The *Plasmodium* under discussion, having erythrocytic stages* resembling those of *P. vaughani* and *P. hexamerium* but agreeing with the former parasite and not the latter in its tendency to invade erythroblasts, is accordingly identified as *P. vaughani*.

The discovery of a strain of this widely distributed species in East Pakistan is not so remarkable as would be that of the North American *P. nucleophilum* or *P. hexamerium*, and the new host, *Sturnus m. malabaricus*, belongs to the same genus

* North American workers had met with little success in their search for E.E. stages in members of the *vaughani* group, apart from a single discovery of a few phanerozoites in the brain of a wild bird positive for *P. hexamerium* and apparently free from other haemosporidians (Manwell, 1951), until Huff's (1956) recent announcement that E.E. schizonts of *P. nucleophilum* occur predominantly in lymphocytes. Laird (1953) described E.E. stages of *P. vaughani* from lymphocytes, macrophages and myelocytes as well as from erythroblasts and normoblasts of *Zosterops flavifrons* subspp. in the New Hebrides, indicative of development of the *elongatum* type. The latter type of E.E. cycle is known among the avian malaria parasites in *P. elongatum* itself, in *P. huffi* (Muñiz *et al* having described such stages from lymphocytes, thrombocytes, granulocytes and macrophages of toucans parasitized by the last-named species) and in *P. nucleophilum*. *P. elongatum*, a small species having elongate gametocytes, bridges the gap between this group and the larger species with their bulky gametocytes and more numerous merozoites. However, the presence or absence of E.E. stages in a particular infection is in itself of little if any systematic importance, for even individual strains of the one species may differ radically in this respect from host to host. For example, Manwell (1951) cited the fact that *P. lophurae* undergoes an E.E. cycle in the turkey, but not in the duck or chicken.

as one of the North American hosts, *S. v. vulgaris* (Hegner and Wolfson, 1938; Thompson, 1943).

SPECIFIC CRITERIA IN THE AVIAN MALARIA PARASITES

As the average merozoite number of species of *Plasmodium* may vary with the strain involved, and is also liable to increase after long-continued laboratory passage in some one particular host (Khabir and Manwell, 1955) and to decrease in the latter stages of individual infections (Manwell, 1949), it is obviously unsound practice to lay too heavy stress upon this factor as a specific criterion in the *circumflexum/lophurae* and *vaughani/hexamerium* complexes.

A low degree of synchronism and a tendency to penetrate mature erythrocytes furnish the best means of distinguishing *P. lophurae* from *P. circumflexum* with its high degree of synchronism and pronounced preference for erythroblasts; and the preference exhibited by *P. hexamerium* for mature red cells affords the chief grounds for the differentiation of this species from *P. vaughani*, which invades both erythroblasts and maturing erythrocytes. At the same time, other species are known to differ within themselves in the matter of synchronism; thus 3 strains of *P. relictum* are known to exhibit a low, high and very high synchronism respectively (authors quoted by Hewitt, 1940).

It has been demonstrated that various species of birds differ widely in their relative susceptibility to strains of *P. lophurae* and *P. circumflexum*, and that although the duck and the turkey are susceptible to blood-induced infection with *P. hexamerium* both birds are refractory to infection with *P. vaughani* (Manwell, 1952). Nevertheless, there is abundant evidence that the complexity of the relations between avian malaria parasites and their hosts is such that particular strains of individual species may react very differently in different birds even to the extent of exhibiting exoerythrocytic schizogony in some hosts but not in others. Reactions to the host may involve morphological change, and Wolfson (1938, 1939) was able to show that when *P. cathemerium* is transmitted to domestic ducks some at least of the gametocytes have dot-like pigment granules resembling those of *P. relictum* in place of the bacilloid granules which characterize *P. cathemerium* in the canary.

It is thus apparent that profoundly unlike patterns of behavior may be awakened in one and the same strain of a species of *Plasmodium* in response to influences attributable to the host, and that these patterns may be reflected by variations from the morphological norm of the species concerned. If the major morphological features of avian malaria parasites as seen in blood films from different hosts and localities correspond closely at all stages, should we, then, attribute greater than varietal significance to the degree of readiness to invade erythroblasts any more than to the degree of susceptibility of a range of laboratory hosts or the degree of synchronism?

Manwell (1949) expressed his conviction that a species of malaria parasite "should exhibit characteristics in the blood film which are sufficiently unique for ready identification if it is to be accepted as valid." It is maintained that it would be very difficult, if not altogether impossible, for any investigator working from unlabelled blood films alone to substantiate an identification of *P. nucleophilum* or *P. hexamerium* (and probably *P. juxtanucleare* and *P. huffi* as well) rather than of the longer-established *P. vaughani*, on the basis of the existing literature. He would be faced with the same difficulty in differentiating between *P. lophurae* and *P. circumflexum*, particularly in cases of chronic infection. Undue "lumping"

of species is as reprehensible as needless "splitting"; for all that, zoological nomenclature having a morphological basis, every effort should be made to integrate fresh material with known species before describing it as new. Where there is close correspondence at all stages of the life history we should be prepared to concede that relatively slight features of difference are of varietal significance rather than allow ourselves to be influenced by the new host and locality into magnifying these features into specific criteria.

Hoare (1955) has recently drawn attention to the unsatisfactory state of our nomenclature of certain parasitic Protozoa, due to the fact that purely biological differences were not taken into account in the older taxonomic terms and concepts. He has therefore proposed a system of classification suited to groups of organisms which, though closely comparable morphologically, are distinct on physiological or ecological grounds. By Hoare's system, where there is no intergrading the organisms are considered as separate species (e.g. *Entamoeba histolytica* and *E. ranarum*); where there is intergrading, the groups are considered as subspecies (e.g. *Theileria parva parva, Theileria parva mutans, Theileria parva dispar*); where there is only minor serological, clinical or host variations they are ranked lower than subspecies and the term "deme" is employed, the demes themselves being characterized as "nosodemes" (on geographical grounds; e.g., Indian, Mediterranean and Sudanese nosodemes of *Leishmania donovani*), "serodemes" (on immunological grounds: e.g. various serodemes of *Plasmodium vivax*) and xenodemes (on host grounds, e.g., xenodemes of *Trypanosoma evansi* from camel, horse and cattle); finally, unstable relapse strains are termed "morphae".

The application of this system to the avian malaria parasites must await the results of further studies, in which Manwell's (1936) suggestion that we recognize as valid species only those plasmodia which will not hybridize with others in the invertebrate host, deserves attention. It is recognized that the strains of *P. circumflexum* and *P. vaughani* described herein may ultimately prove distinct enough to merit subspecific rank, but it is not considered that any useful purpose would be served by the designation of new subspecies in the present state of our knowledge.

SUMMARY

Of 262 birds of 27 species examined for haematozoa in East Pakistan, 22 examples of 6 species proved to be infected. A strain of *P. circumflexum* differing from the typical one in forming only 10–26 merozoites (av. 14) instead of 13–30 (av., 19.8 ± 5) is described from 4 (of 76) examples of the Common Indian Mynah, *Acridotheres tristis tristis* (L.), and 5 (of 42) examples of the Indian Grey-headed Mynah, *Sturnus malabaricus malabaricus* (Gmelin); and a strain of *P. vaughani* that most commonly forms 6 merozoites is recorded from 2 of the *A. t. tristis* and 7 of the *S. m. malabaricus*. The Indian Spotted Dove, *Streptopelia chinensis suratensis* (Gmelin), and the Indian Jungle Mynah, *Acridotheres fuscus fuscus* (Wagler) are laboratory hosts for *P. circumflexum*. Five species of birds were found to be parasitized by *Haemoproteus* spp., but none of the remaining genera of avian haematozoa were recorded. Specific criteria in the avian malaria parasites are briefly discussed, attention being drawn to the very close morphological resemblances between certain of the accepted species and the likelihood that some of these will prove to be no more than subspecies or strains of species described earlier. It is pointed out that the use of phase contrast lighting renders visible fine structural details which can no longer be made out by bright field methods in faded, Giemsa-stained blood films.

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EXPLANATION OF PLATES

PLATE I

Plasmodium circumflexum Kikuth, from *Acridotheres t. tristis* (L) and *Sturnus m. malabaricus* (Gmelin)

FIGURES 1 and 2. Erythroblasts recently invaded by merozoites (*Sturnus*).
FIGURE 3. Signet ring trophozoite in normoblast (*Sturnus*).
FIGURE 4. Amoeboid trophozoite in erythrocyte (*Acridotheres*).
FIGURE 5. Binucleate schizont (*Acridotheres*).
FIGURE 6. Hexanucleate schizont (*Sturnus*).
FIGURE 7. Schizont, nuclear division into 10 taking place (*Acridotheres*).
FIGURE 8. 14—nucleate schizont (*Acridotheres*).
FIGURE 9. 16—nucleate schizont (*Sturnus*).
FIGURE 10. 26—nucleate schizont (*Sturnus*).
FIGURE 11. Double infection, schizonts (*Acridotheres*).
FIGURE 12. " " " (*Sturnus*).
FIGURE 13. " " " (*Acridotheres*).
FIGURES 14–17, 18. Segmenting parasites producing 10–20 merozoites (*Sturnus*).
FIGURE 17a. Merozoites free in plasma (*Sturnus*).
FIGURE 19. Macrogametocyte partly surrounding host cell nucleus (*Acridotheres*).
FIGURE 20. Macrogametocyte completely surrounding host cell nucleus (*Acridotheres*).
FIGURE 21. Microgametocyte completely surrounding host cell nucleus (*Sturnus*).
FIGURE 22. Microgametocyte completely surrounding host cell nucleus (*Acridotheres*).

PLATE II

Plasmodium vaughani Novy and MacNeal, from *Sturnus m. malabaricus* (Gmelin)

FIGURES 23 & 24. Erythroblasts recently invaded by merozoites.
FIGURES 25 & 26. Signet ring tropohozoites in normoblasts.
FIGURE 27. Amoeboid trophozoite, showing refractile granule.
FIGURE 28. Older trophozoite.
FIGURES 29 & 30. Quadrinucleate schizonts.
FIGURES 31–35. Hexanucleate schizonts.
FIGURES 36–38. Octonucleate schizonts.
FIGURE 39. Double infection, trophozoite and segmenter.
FIGURE 40. Triple infection, trophozoite and 2 schizonts.
FIGURE 41. Production of 8 merozoites.
FIGURES 42–45. Macrogametocytes.
FIGURES 46–48. Microgametocytes.

PLATE I

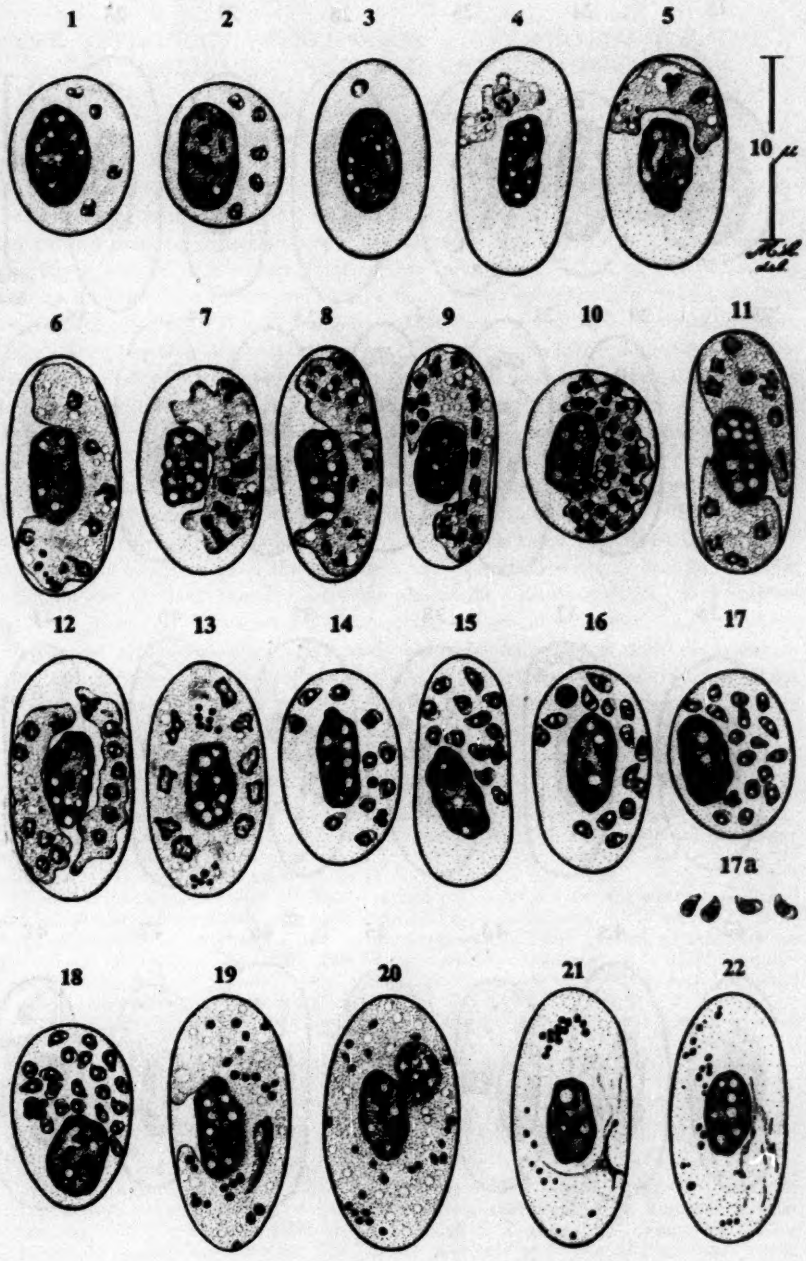
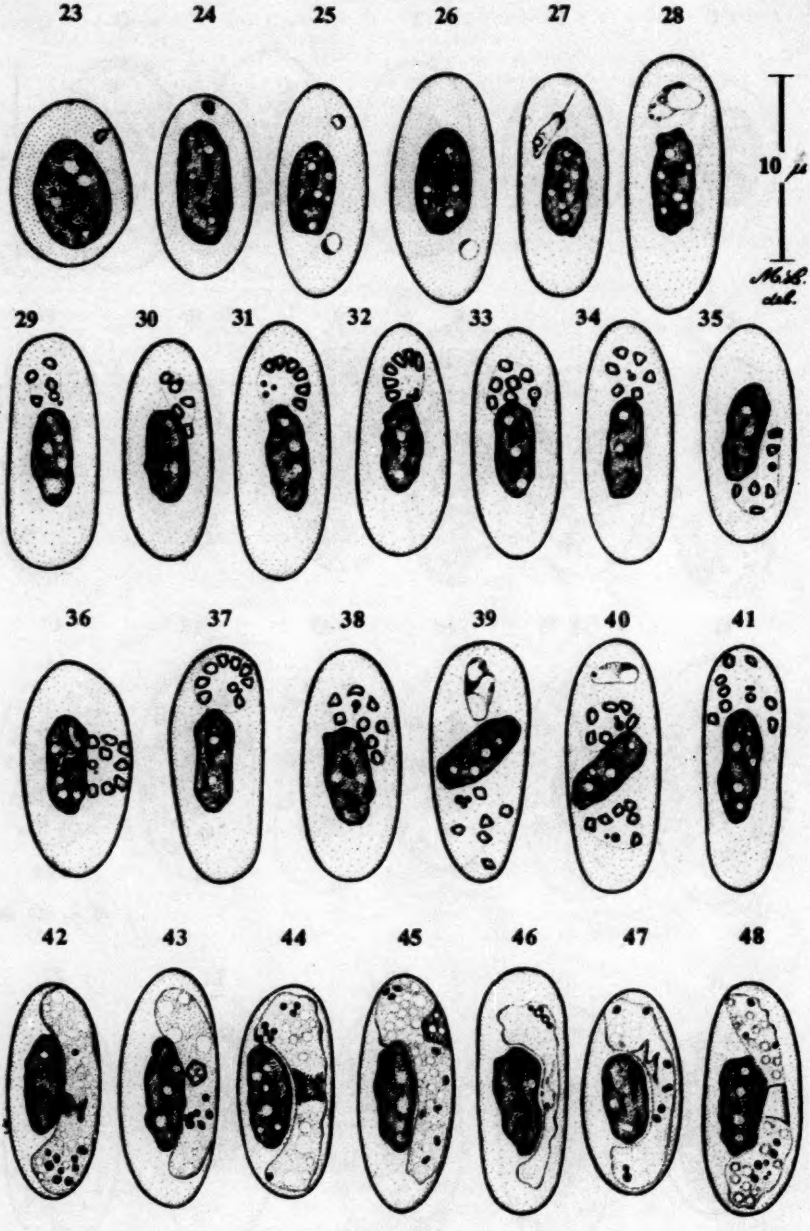


PLATE II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THE REPRODUCTIVE BEHAVIOR OF *PLASMODIUM CATHEMERIUM* IN DUCK AND CHICK EMBRYOS

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The questions of immunity and periodicity of reproduction of the malaria parasite have occasioned considerable interest in recent years. It is common knowledge that certain malaria organisms show a characteristic and definite synchronism in their reproduction. It has been suggested by Taliaferro (1925) that this periodicity is the result of an inherent tendency in the parasite to reproduce to a certain rhythm. Boyd (1925, 1929, 1933) and Stauber (1939) have shown that this inherent tendency of the parasite is profoundly affected by environmental conditions. All attempts to solve the problem of the relation of environmental factors to periodicity of parasitic reproduction have been seriously hampered, however, by the impossibility of securing completely controlled environmental conditions in the vertebrate host.

If a host could be found in which the malaria parasite could successfully establish itself and whose environment is subject to a minimum degree of fluctuation, a closer approach could be made to the relation which these influences surrounding the host bear to activities of the parasite itself. In an attempt to find such a host bird embryos have been used for the avian plasmodia. Wolfson (1940) and Stauber and Van Dyke (1945) succeeded in obtaining an infection in duck embryos with *Plasmodium cathemerium*. In this study we have made use of their discovery to study the parasite's behavior, particularly with regard to reproduction, in a host whose environment normally remains more or less constant in relation to such factors as temperature, light, food supply, etc.

METHODS AND MATERIALS

The $3H_2$ strain of *P. cathemerium* which was obtained through the kindness of Dr. Clay G. Huff and which was maintained by continuous passage in female canaries in this laboratory was used. For comparison of infections in chick embryos the 12a strain of *P. lophurae*, the 6H strain of *P. circumflexum* and the 9a strain of *P. gallinaceum* were used.

P. cathemerium studies were principally carried out using as hosts the embryos of Khaki Campbell Ducks (obtained locally), while embryos of white leghorn chicks served as hosts for the comparison of infections of the various plasmodia in other animals. Duck embryos were injected at 14 days of age while those of the chick were inoculated at 10 days of incubation. The procedure for inoculation and the technic of making blood films were essentially the same as those described in previous papers (McGhee 1950).

All blood films were stained with Giemsa's stain and all figures of counts contain no more than a 15% error.

EXPERIMENTAL RESULTS

The adaptation of P. cathemerium *to continuous passage in duck embryos.*

As was true in chick embryos (McGhee, 1954) canary erythrocytes were toxic

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A portion of this work dealing with periodicity was begun while the author was a graduate student. Results expressed herein, however, represent experiments in which more refined technics were applied. The author is indebted to Dr. George H. Boyd for the original idea and for his aid during its early development. The work is supported in part by United States Public Health Grant E924.

to duck embryos and injections of blood from a canary with an infection of 15 parasites per 100 erythrocytes resulted in the death of 8 out of 10 embryos. Because of the high mortality rate and the resultant decrease in the number of host animals blood films were made at intervals of 4 days rather than daily. At the end of 8 days one embryo had a parasitemia of 200 parasites per 10,000 duck erythrocytes, while the other was negative. Two-tenths ml of blood from this embryo was injected into four 14-day embryos and again blood films were made at 4-day intervals.

Starting the 2nd day of the passage daily blood films were made (Fig. 1).

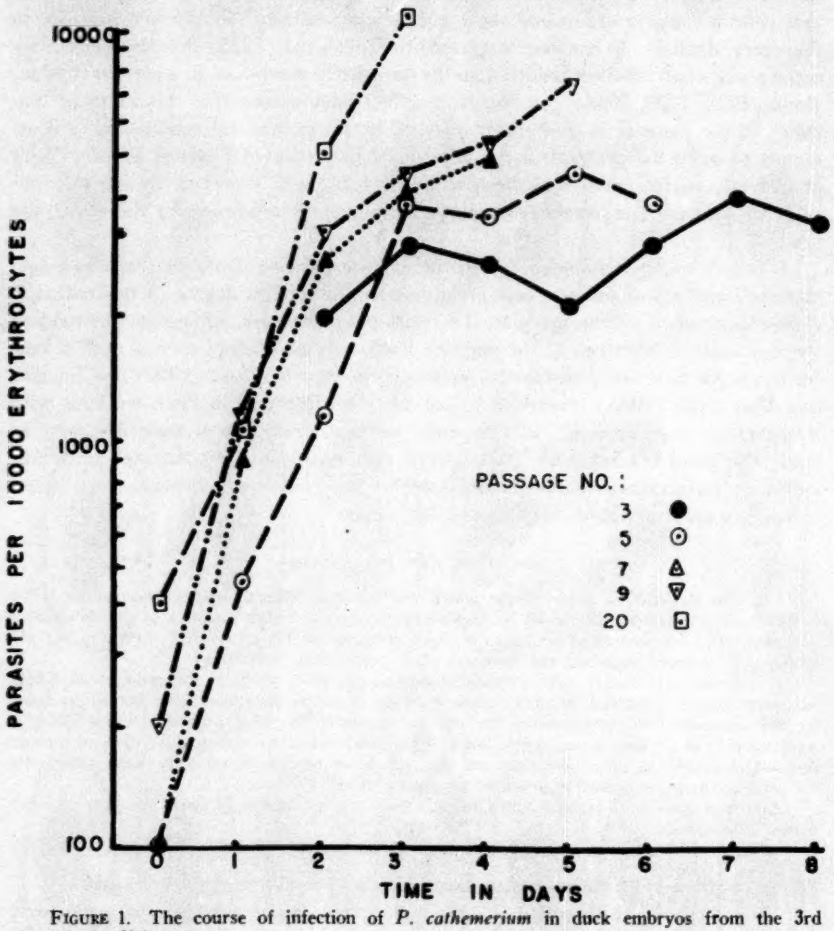

FIGURE 1. The course of infection of *P. cathemerium* in duck embryos from the 3rd through the 20th passages.

On day 2 a parasitemia of 1200 parasites per 10,000 erythrocytes was found. From that day until the 5th day no real increase or decrease in the number of parasites was noted, although there was a slight rise on the 7th day with a decline on the 8th. All embryos were sacrificed for transfer.

The number of parasites increased in the 5th passage and during subsequent passages until on the 20th passage there was almost a straight line increase as shown on the semilogarithmic graph (Fig. 1). There was a marked difference in the rate of increase of parasites from the initial through the 9th passage in the duck embryos. Each succeeding passage was marked by the more rapid increase in number of parasites until on the 20th passage the average parasitemia on the 4th day was over 10,000 parasites per 10,000 erythrocytes.

Infection of chick embryos with P. cathemerium *and a comparison with other plasmodia in the same host.*

Initial attempts to secure patent infections of *P. cathemerium* in chick embryos were unsuccessful. The parasitemia in the donor canaries was small (15 parasites per 100 erythrocytes) and inasmuch as there was wholesale destruction of canary red cells, it was believed that better success could be obtained by using blood of parasitized duck embryos for infecting doses.

Blood from a duck embryo containing 4000 parasites per 10,000 erythrocytes was injected intravenously into six 10-day old chick embryos. Blood films were made soon after injection and daily thereafter. The results, as shown in Figure 2, represent average daily counts on all embryos.

Initial infections averaged 600 parasites per 10,000 erythrocytes. On day 1 the number of parasites had increased to 1,000. On subsequent days there was an irregular but rather constant decline in the number of parasites, but they could still be found 10 days thereafter (or on the expected day of hatching). None of the embryos hatched.

Several attempts were made to subpassage from infected embryos, but in no case was there any indication of the parasite's becoming better adapted to the chick embryo. It was possible to find a few parasites up to 5 days after transfer, but they were never numerous enough to count with any validity.

For comparison, established infections of *P. lophurae, P. gallinaceum* and *P. circumflexum* were studied and average infections used to construct graphs of the curve of parasitemia (Fig. 2). Infections with *P. lophurae* and *P. gallinaceum* have already been described and follow a line similar to that of established infections of *P. cathemerium* in duck embryos. *P. circumflexum* was established in both chick and duck embryos from infections in canaries. No attempt was made to determine if the same period of adaptation was necessary but established infections in either chick or duck embryos were similar to *P. gallinaceum* and *P. lophurae.*

Periodicity of P. cathemerium *in duck embryos.*

To ascertain whether *P. cathemerium* was subject to a periodic development of merozoites in duck embryos, blood films were made at 2-hour intervals from the injection of parasitized cells through 54 hours. Blood films were begun at noon and continued throughout the night.

Although there was some slight indication of a peak of segmentation occurring at or around 6 in the evening in certain embryos (Fig. 3—D, F, G, H) the number of segmenting parasites was in the main irregular and few in number. Twenty-four hours after injection there was in all embryos the beginning of a sharp increase in numbers of segmenters. The increase was rapid and reached a peak between 8 and 12 in the evening. In certain embryos (Fig. 3—C and F) the percentage of mature schizonts was only about 12 to 15 while in others (Fig. 3—A and H) as

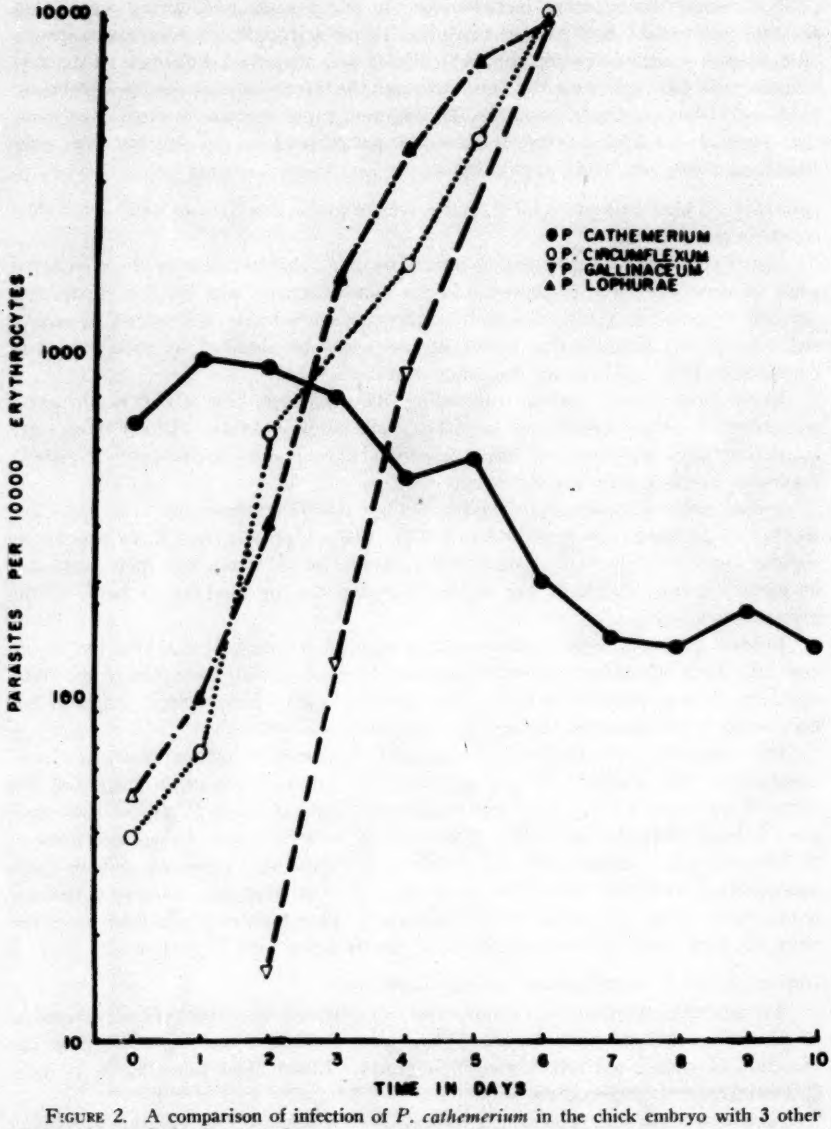

FIGURE 2. A comparison of infection of *P. cathemerium* in the chick embryo with 3 other species of malaria in the same host.

many as 36% of the parasites were undergoing segmentation. Following the peak there was a return to the low level observed prior to the evening peak of segmentation.

Although additional films were made through 8 P.M. of the next day there was never any evidence of a second peak. Most of the embryos were dead on the 4th

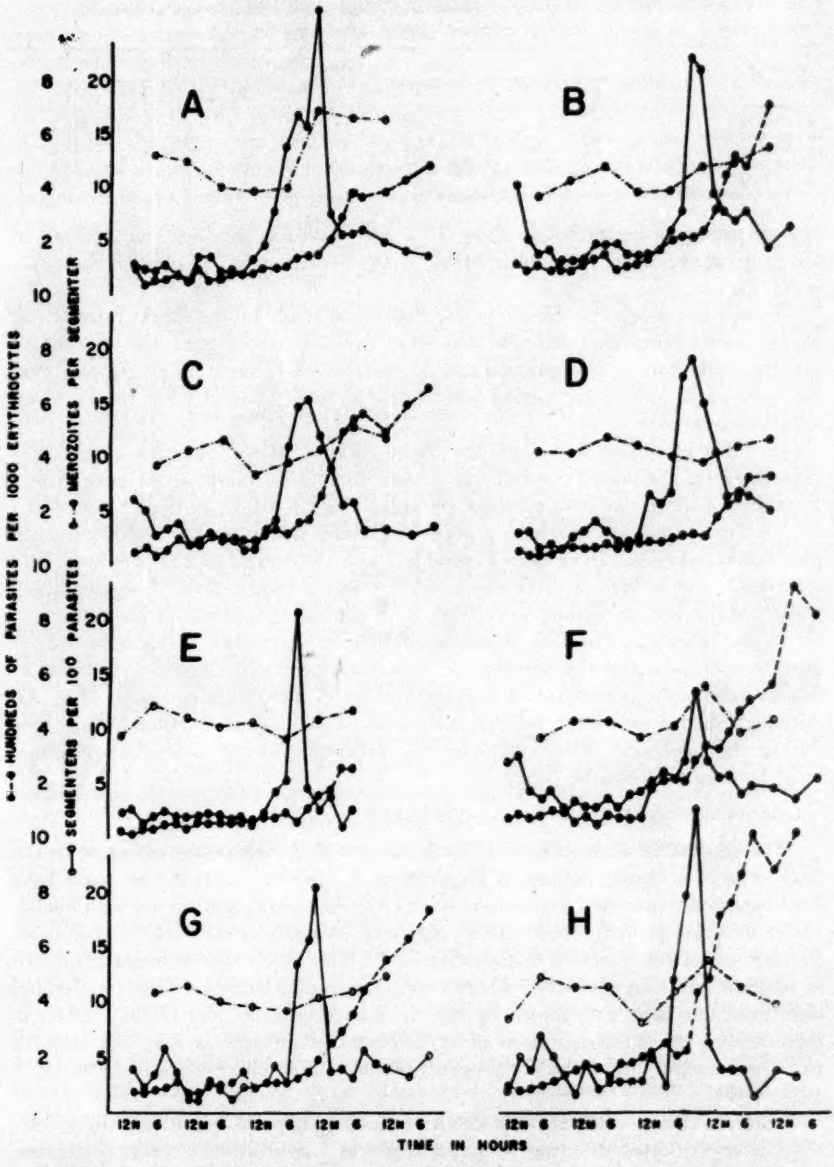

FIGURE 3. The periodic rate of reproduction of *P. cathemerium* in duck embryos and the bi-hourly course of infection in each embryo. Included are merozoite counts of parasites from each embryo.

day but in certain other heavily infected individuals bi-hourly blood films were made and indicated no evidence of an additional peak (Table I). Indeed, the number

TABLE I.—*The percentage of segmenters of* Plasmodium cathemerium *in heavily infected embryos after 4 days' infection*

Time of Day	Embryos			
	I	J	K	L
8 : 00 A.M.	7.5	9.7	12.3	10.2
10 : 00 A.M.	8.2	14.5	10.4	11.3
12 : 00 Noon	6.6	9.7	12.2	15.9
2 : 00 P.M.	10.0	15.2	12.6	17.7
4 : 00 P.M.	6.4	8.5	9.7	14.7

of segmenting forms leveled to about 10% and stayed at this point until death of the embryo, indicating a complete break in any synchronous behavior of the parasites (Fig. 2).

From the above experiments it was concluded that: 1) the initial injection of parasites into uninfected embryos was from embryos whose parasites were in an asynchronous state of reproduction and 2) there was a momentary return to a synchronous cycle after a lag period which was in turn followed by a return to an asynchronous state.

Included in Figure 3 is a graphic representation of the numbers of parasites present during the period covered. In general there was, following the peak of segmentation, a sharp rise in numbers of parasites (Fig. 3—B, C, F, G, H) but in embryo A the rise was not nearly so pronounced, rising only from 200 to 400 parasites per 10,000 red cells. In embryo E even less rise (from 150 to 200 parasites) was recorded. Yet in both of these embryos there was a precise time of segmentation. Clearly there was something interfering with the usual increase of parasites.

In the belief that this lack of increase following segmentation might be due, in part, to a reduction of the numbers of merozoites produced, counts were made at 4-hour intervals to determine the average number of merozoites produced (Fig. 3). Although fluctuations were present there was no significant decrease at any time during the infection. The reason for the difference in the courses of infection must be sought elsewhere.

DISCUSSION

The process of adaptation of *P. cathemerium* to a continuous existence in the duck embryo is clearly defined in Experiment 1. Indications that this might have been expected were noted in previous work (McGhee 1949) and shown with finality in the infection of baby mice with *P. lophurae* (McGhee 1951). It is reasonable to assume that the injection of parasites into a host which was different from that in which it had long sojourned would necessitate some changes. That a difference does exist has been well shown by the work of Rappaport and Guest (1948) in their studies on the constitution of erythrocytes of animals and by the author's work on susceptibility of the erythrocytes of different species (McGhee 1950, 1953, 1954, 1956).

Thus the change of hosts could result in the malaria parasite's attempting to survive in erythrocytes differing in physiochemical constitution to varying degrees. In the mouse *P. lophurae* had to be carefully nursed to allow it to establish itself in this host, while *P. cathemerium* was able rather quickly to adapt itself to the duck red blood cells. In the chick embryo, however, the ability of *P. cathemerium* to adapt itself to the erythrocytes was taxed beyond its capabilities with the result that a transfer from infected to uninfected embryos diluted the numbers of parasites

to a position that it was unable to increase enough to overcome the deficit. *P. gallinaceum, P. circumflexum* and *P. lophurae* quickly adjusted and in a very short period overwhelmed their host.

Upon casual observation of the environmental conditions prevailing in the developing duck embryo, all factors such as heat, light, humidity and feeding appear to be fixed. If left in the incubator this would be true, but experimentation upon the embryos must of necessity force some temporary change, slight or not, upon the exogenous forces surrounding them. Apparently these changes effected no significant influence on the synchronism of reproduction.

Movement of the embryo during the time of infection is confined to gentle rocking occasioned by the muscular contraction of the amniotic wall. The effect of light can be regarded as slight, although a certain amount of it could penetrate to the embryo during the withdrawing of blood. Removing blood from the host may also exert an influence upon the parasite.

Even if outside influences were controlled certain conditions might still have to be considered. Physico-chemical conditions of the embryonic tissues may in themselves affect the course of infection. There may be physiological cycles of the embryo as well as of the parasite which could act directly on the activity of the parasites. Notwithstanding all these factors which may prove undesirable in the determination of the inherent potentialities of *P. cathemerium* it was found that definite synchronism of reproduction did exist. This factor in itself would tend to minimize the importance of the uncontrolled conditions.

It would appear that the genetic constitution of the parasite exerts a profound influence upon its reproductive cycle and that environment acts as a limiting or accelerating effect on this inherent force.

In certain of the embryos the parasites, although displaying a sharp peak of segmentation, failed to show the expected increase in parasites following this period of segmentation. The failure to achieve the expected parasitemia is clearly not due to a reproduction-inhibiting antibody but rather reflects a resistance of a parasiticidal nature. The view formerly held by this author of the complete susceptibility of the embryo to malaria (once infection is established) can no longer be considered feasible. The nature of the resistance is yet to be determined.

SUMMARY

The change from the canary to the duck embryo necessitated adaptive changes on the part of *P. cathemerium* (and perhaps other plasmodia as well). After a few passages, however, the course of infection was rendered similar to that seen in *P. gallinaceum* and other malarias in chick or duck embryos. Attempts to establish the parasite in chick embryos were unsuccessful.

P. cathemerium in duck embryos displayed synchronism of parasitic reproduction through 1 schizogonous cycle, but the periodicity collapsed before a second peak could be reached. In certain embryos the expected increase in parasite numbers following peaks of segmentation was not realized. No significant deviation of merozoite numbers was observed and it was concluded that the supposed lack of resistance in embryos was in all probability not true.

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REPRODUCTION OF *TOXOPLASMA GONDII* BY INTERNAL BUDDING

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Toxoplasma gondii was described originally as dividing "par bipartition" (Nicolle and Manceaux, 1909). In a careful study of the cytology of *T. gondii*, Cross (1947), using iron hematoxylin, Giemsa and Feulgen staining techniques, depicted forms of the organism which she interpreted as being part of a cycle of binary fission. Chernin and Weller (1957) observing, as other workers had done, the regularity with which parasites were found in multiples of 2 within infected tissue culture cells, also considered division of *T. gondii* to be by binary fission.

Early in the course of our studies on internal morphology of *T. gondii* stained with silver protein, structures were seen which had not been described before for this species. Upon further examination it became clear that these structures were daughter cells developing within parent organisms. Instead of dividing by binary fission, as had been previously supposed, *T. gondii* appeared to reproduce by a type of internal budding in which each parent produced 2 daughter cells, the parent being destroyed in the process. It is the purpose of this report to describe this division in some detail. Although many morphological features were seen on the silver-stained smears, only those points which are pertinent to the division process will be described here. A subsequent paper will deal in greater detail with the anterior cone, an internal rod, and an external fiber that were seen.

MATERIALS AND METHODS

The silver-protein method that gave best results was basically that recommended by Honigberg and Davenport (1954). In some cases time of development in hydroquinone was varied, and some smears were not toned in gold chloride. Commission certified "Protargol S" (Winthrop-Stearns) was used for most preparations, but an old sample of strong medicinal Protargol (Winthrop) also gave good results.

In common with other workers, we found that silver staining was erratic and unpredictable. Attempts to control results by manipulating pH, time or temperature were not successful. By varying the degree of gold toning some control over density was possible but only if the initial silver impregnation was successful. No amount of toning could improve a very lightly impregnated smear; conversely, toning could be entirely omitted if the silver had been laid down in sufficient density.

The general method of preparing smears for staining was as follows: Peritoneal exudate was drawn from mice infected with *T. gondii* 4 or 5 days previously, and was discharged into about 10 volumes of 0.1 or 1.0% formol-saline. After 10 minutes the suspensions were centrifuged and smears were prepared from the sediment. Before the smears were completely dry, they were put into Bouin's or Hollande's fixatives and left for 2 or more days. The smears were then transferred to 70% ethanol for a few hours. If they were not stained immediately they were left in alcohol in the refrigerator until needed Modifications of this fixation method consisted of exposing drops of saline-washed toxoplasmas to formaldehyde vapor before fixation in Bouin's or Hollande's, or use of osmium vapor as the sole fixative. In the latter case it was possible to stain smears with silver directly; with all other fixatives, use of a $KMnO_4$ bleaching solution before the silver bath, as described by Honigberg and Davenport, was absolutely essential to bring out details of structure.

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Wet mounts of living or formalin-washed toxoplasmas were examined briefly with anoptral phase (Reichert) and with the interference microscope (AO-Baker), but no structures were seen other than those visible with conventional optics. Dark field observation of stained smears was useful sometimes in showing up details of structure which were less clear in bright field.

RESULTS

The appearance of various internal structures differed somewhat depending upon the fixation as well as upon the staining. In general, division stages were more clearly defined following Bouin's fixation; other structures were brought out better by Hollande's fluid. The descriptions that follow are a composite of observations made on the different types of smears.

General Morphology

The "resting" cell possessed a distinctive type of nucleus (Figs. 1–3, 29–35). It appeared as a large, well-defined, irregularly rounded or oval mass, at least 1 side of which was frequently flattened so that some of the contours were angular. With light staining and with some types of fixation, about 5 or 6 small granules could be seen scattered irregularly in the less darkly stained nucleoplasm. In addition, 1 prominent, larger and more darkly stained body was always present. On some smears the latter appeared to be solid but in delicate preparations it seemed to be a very thick-walled, lobed, doughnut-shaped structure in which the center perforation could frequently be seen (Plate II, Fig. 8). This endosome occurred most frequently within the nucleoplasm in contact with some part of the nuclear membrane, but occasionally it was seen to lie altogether outside the nucleoplasm (Figs. 1, 7, 29–35). In darker preparations the smaller granules became progressively less differentiated from the nucleoplasm, but the endosome remained distinct in all except the darkest smears. In those, the entire nucleus assumed the character of a homogeneous, opaque mass with no detectable fine structure.

The anterior portion of the cell, from just behind the pointed end to about 1/3 or 1/2 the distance to the anterior margin of the nucleus, frequently stained almost as darkly as the nucleus. Because of the tapered shape of the organism, this stained area looked like a truncated cone, but only the blunt apex of the cone seemed to have a definite morphology. In "head-on" view the apex appeared as a distinct ring with strong affinity for stain (Figs. 4, 18, 24, 34, 35). In profile, the perforation of the ring was only faintly discernible in bright field but was clearly visible in dark field (Figs. 8, 16). The sides of this anterior cone did not always stain as darkly as the apex and the precise morphology of this region is not yet clear.

The pointed end of the organism, anterior to the apical ring, was often not visible because of the low affinity shown by the cytoplasm for the stain. However, in those preparations where the cytoplasm did stain with silver, and in the rare cases where a counterstain was tried, the relationship of the cone to the anterior tip was clear (Figs. 12, 27, 29, 30).

One more structure seen with great regularity was a mass or clump of granules at the very posterior end of the organism (Figs. 1, 2, 3, 5, 29, 30, 32 and others). Very often the cytoplasm behind the nucleus was only faintly stained but the posterior mass with its strong affinity for silver served as an indication of the length of the cell.

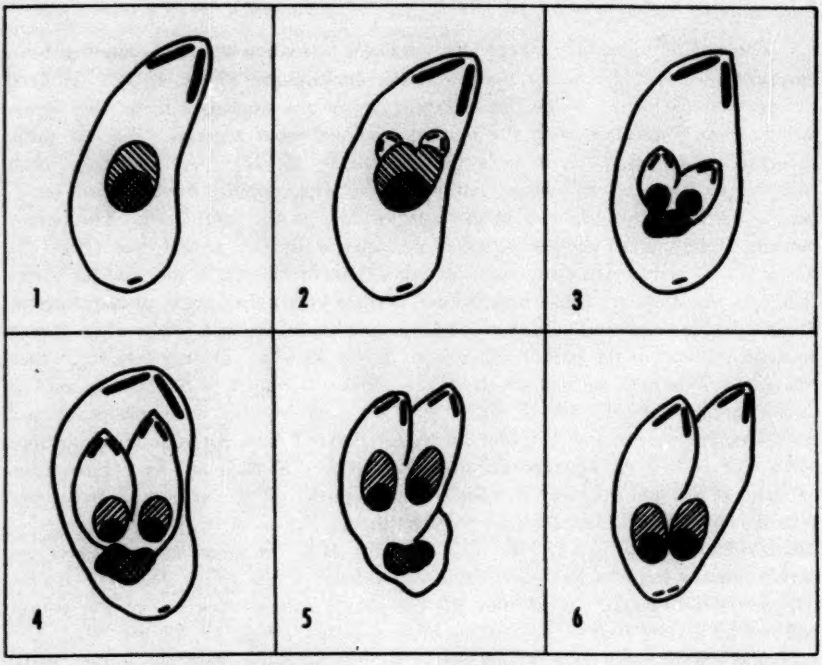
Reproductive Stages

The earliest indication of reproduction is the formation of 2 dark-staining, bud-like processes on the anterior margin of the nucleoplasm (Figs. 4, 5). At first, the projections appear to be direct extensions of the nucleus but, as they grow larger, their connection with the nucleus becomes more tenuous (Fig. 6) until, finally, no connection can be seen (Figs. 7, 8, 9, 36, 37). At this stage, each daughter cell appears to consist entirely of an argyrophilic, dome-shaped structure which is a small duplicate of the anterior cone of the parent cell. The parent nucleus, including the endosome, shows no signs of division at this time (Figs. 36, 37, 38). Growth of the daughter cells takes place primarily in the anterior direction. As the structures push forward and become larger they begin to take form as distinct bodies, each with its own limiting membrane, so that finally they appear as small replicas of the parent cell (Figs. 10–19, 39–41). During this stage each cell acquires its own nucleus which also is a reduced replica of the resting nucleus of the parent (Figs. 13, 16, 17, 41). It is not clear whether the nucleoplasm and endosome portions of the daughter nuclei are derived from corresponding portions of the parent cell, or whether differentiation occurs in each cell by condensation of some of the nucleoplasm that each has acquired. The parent cell frequently retains endosome and nucleoplasm portions throughout most of the development of the daughter cells (Figs. 15, 16, 17, 19, 20, 39, 41). In some cases, however, no parent nucleus can be seen in advanced reproductive stages (Figs. 18, 40). As the daughter cells become larger they fill the entire anterior portion of the parent cell and all 3 cone regions are pushed close together (Figs. 17, 18, 19, 40). The two new cells subsequently break out of or are liberated from the parent while still attached to each other (Figs. 20, 21, 22, 42, 43), and complete their division outside. During the process of breaking out, the parent cell is apparently destroyed. Paired cells frequently appear to be shorter than other cells in the vicinity but no measurements have been made to confirm this subjective impression. Text-figure 1, is a schematic representation of the division process as we interpret it.

A few attempts were made to stain smears with a modified Feulgen method. Results were similar to the figures presented by Cross (1947). Except for the nucleus, none of the argyrophilic structures were stained by the Feulgen reagent and no indications were seen of the method of reproduction which we have described.

In the above description an attempt has been made to fit together into a rational pattern observations made on hundreds of organisms. This has necessitated ignoring some cells where the distribution of nuclear and daughter cell material did not fit the pattern (Figs. 23–28, 44, 45). In these "anomalous" cases, daughter cells appeared to be developing in opposite directions (Figs. 23, 24, 25), at right angles to each other (Fig. 26) or to the parent nucleus (Fig. 44), or the cell contents were a complete jumble in which it was not possible to separate daughter cells from parent structures (Fig. 27). In 2 instances, daughter cells appeared to be developing (Fig. 45) or emerging from the posterior end of the parent (Fig. 28).

In order to gain some idea of the frequency with which organisms occurred in the various stages of reproduction and as anomalies, an arbitrary classification was set up and the number of cells occurring in each stage was counted. About



TEXT-FIGURE 1. Schematic representation of the division process in *Toxoplasma gondii*.

500 free-lying organisms were counted on each of 3 smears prepared from exudates obtained from different mice on different days. The results are shown in Table I. All 3 smears yielded similar percentages of organisms in the various stages. About 14% of all cells were in some stage of division, and most of these (10.8%) were in the early stages before the daughter cells showed separate nuclei. The percentage of organisms containing daughter cells with separate nuclei was 2.3, and that of dividing forms outside the parent cell but still joined together was 1.0. A little less than 1% of all organisms seen could not be readily interpreted according to the pattern of reproduction we have postulated.

TABLE I. *Distribution of toxoplasmas according to stage in the division process.*

Slide	Resting stage[1]	Early division[2]	Medium division[3]	Late division[4]	Anomalies[5]	Total
I. 12/2/55	402(80.1)[6]	70(13.9)	17(3.4)	7(1.4)	6(1.2)	502
II. 12/18/55	453(87.9)	49(9.5)	8(1.6)	3(0.6)	2(0.4)	515
III. 1/4/56	443(86.8)	46(9.0)	10(2.0)	6(1.2)	5(1.0)	510
TOTAL	1298(85.0)	165(10.8)	35(2.3)	16(1.0)	13(0.9)	1527
			216(14.1)			
Breakdown of division stages only, in per cent		76.3	16.1	7.6		

[1] No sign of daughter cells or of nuclear changes (figs. 1–3).
[2] From earliest appearance of daughter "buds" up to, but not including, separate nuclei for each daughter cell (figs. 4–11).
[3] From separate nuclei for each daughter cell through any stage still within the parent cell (figs. 12–20).
[4] All cells still joined together but lying outside parent cells (figs. 21–22).
[5] Unusual configurations of daughter and parent cells (figs. 23–28).
[6] Figure in parenthesis is percentage of all cells counted on a particular slide.

Because of the special interest which attaches to the demonstration of a flagellum or similar structure in *Toxoplasma*, attention is called to Fig. 27 which shows a distinct spike-like extension at the anterior end of the cell. A detailed account of this and other organelles will be presented in a later paper, but at this point it should be mentioned that long, thread-like appendages were regularly demonstrable in organisms treated with dilute formalin. The staining of these threads with fluorescein-labelled antibody has already been reported (Goldman, 1957).

DISCUSSION

Although internal budding is characteristic of Suctoria and Myxosporidia, the process in those groups differs considerably from that seen in *Toxoplasma*. Some important distinguishing features of internal budding in *Toxoplasma* are: (1) The most prominent somatic feature of daughter cells, the dark-staining cone, appears to derive directly from the undivided parent nucleus; (2) there is no indication that the parent nucleus is the direct source of daughter cell nuclei; (3) daughter cells resemble the parent in all morphological aspects except size; (4) nothing resembling a brood chamber can be detected; (5) characteristically, 1 parent yields 2 daughter cells.

Because this process is so different from methods of reproduction described in the past for protozoa, the term *endodyogeny*[1] is proposed for it. So far, the only other species shown to divide by a similar process is *Besnoitia jellisoni* (Goldman, et al, 1957), a parasite similar to *Toxoplasma* in other respects as well.

It is pertinent to consider at this point why this method of reproduction has escaped detection in the past, in view of the attention which *Toxoplasma* has received since it was first described. The answer to this lies quite clearly with the fact that the anterior cone organelle has either not been seen before, or, if seen in electron micrographs, its significance has not been recognized. If, in the photographs presented here, the staining of parent and daughter cone structures is ignored and only nuclear changes are observed, the appearance, to all intents, is one of binary fission. It is only by rendering visible the changes taking place within the cytoplasm that it becomes obvious how different the process is from classical binary fission. As far as we can determine, none of our observations contradict those made previously as to the gross changes which take place when 1 toxoplasma reproduces to form 2. The description by Pulvertaft *et al* (1954) of the division of living toxoplasmas observed with the phase microscope, is completely applicable to our material with the exception that their description does not tell as much of the story as can be seen in silver-stained smears.

The question of the taxonomic position of *Toxoplasma* has received considerable attention in recent years (Van Thiel, 1956, Westphal, 1954, contain extensive bibliography). Structures revealed by electron microscopy suggest affinity between *Toxoplasma* and *Sarcocystis* (Ludvík, 1956), but it has been difficult to relate either genus to any of the established classes of protozoa. The method of reproduction described here makes it improbable that *Toxoplasma* can be considered a modified member of the Trypanosomidae (Westphal, 1954), or Eugregarinida (Van Thiel, 1956) unless, of course, it can be shown by techniques sim-

[1] *Endodyogeny* is derived from three Greek words: *endon*, meaning within or inside, *dyo* meaning 2, and *genesis*, meaning birth. Its use here denotes a type of reproduction in which 2 individuals are formed within a parent cell.

ilar to those used here, that this type of reproduction occurs in those groups too. For the present it seems best to consider *Toxoplasma, Besnoitia* and *Sarcocystis* together and separate from any established class of protozoa. Since silver-protein staining is readily available in practically any laboratory, and since the morphological features demonstrated in *Toxoplasma* by this method are so distinctive, it should not be difficult to assemble enough morphological data on other species to make possible a rational location of this group in the Protozoa.

The morphology of the nucleus in silver preparations differs considerably from that shown by hematoxylin, Feulgen or Giemsa stains, but is quite similar to that described and figured by Gustafson *et al* (1954) on the basis of electron microscopy of thin sections. The consistent presence of a large dense mass in the nucleus, which was not similar to the type of structure seen in Bouin-iron hematoxylin preparations, was noted by these authors, and their photographs show that the location of this mass within the nucleus was not uniform. This agrees well with our observations on what we have called the endosome. Since this mass is Feulgen-negative and is not basophilic in hematoxylin preparations, and since its relation to the other granules of the nucleus and to the nucleoplasm seems quite irregular, it would seem best not to use the terms "karyosome" or "nucleolus" in referring to it. Further work is needed to elucidate its significance.

Gustafson *et al* described lobed, elongated and horse-shoe shaped nuclei whose interpretation was obscure, but which they thought might be division stages. It is quite possible that they actually were seeing sections of dividing nuclei and developing daughter cells, but because of the problem of orientation in thin sections, the total picture of what was taking place was not clear. It is certainly conceivable in the light of our observations that their figures 13, 15, and 16 were early stages in the development of daughter cells.

Bringmann and Holz (1954), Ludvík (1956), and Meyer and Mendonça (1957), working with electron micrographs of toxoplasmas, have described an anterior ring-like organelle with associated fibrils. This organelle seems to be identical with what we have called the apical ring of the anterior cone. In our figure 1, fibrils associated with the ring are clearly shown. The "conoid" of Gustafson *et al* seems to be analogous to the perforation of the apical ring (figs. 8 and 16). The published electron micrographs of these authors reveal a dense line covering the anterior end of the toxoplasmas in almost every section showing the conoid in profile (Gustafson *et al* figs. 2, 4, 7, 12, 13 and 15). It seems likely that this represents in section the anterior cone structure. Van Thiel (1956) was able to see in one organism studied with the electron microscope a structure which he called "conus". On the basis of size, description and text drawing, this "conus" appears to be what we have called the anterior cone. Speculations as to the function of these anterior organelles have been indulged in by the other authors mentioned and we have nothing to add at this point to clarify this problem.

Cross (1947) states that "two granules have been found fairly constantly between the nuclear matrix and the broader end of specimens that were stained with Giemsa's or Heidenhain's iron methods", but since these granules are not shown in her figures it is not clear whether she was seeing our "posterior mass" or some other structures. There is little information on which to base speculation concerning the function of this vaguely defined and minute structure. Possibly, the

posterior mass may represent part of the nuclear endosome from the parent cell. Although it is frequently difficult to be sure of the exact topography of this region, the posterior margins of growing daughter cells seem to extend to the level of the residual parent nucleus (figs. 15, 16, 17, 19, 20, 39, 41). Such an origin would be consistent with the strong argyrophilia of the mass.

Although the percentage of dividing forms found on fixed slides gives no indication of the absolute rate of reproduction, the frequency with which organisms are found in any one stage of a continuous process may be an indication of the relative rates at which the various stages proceed. On this hypothesis, it appears that the early development of daughter cells is the longest portion of the division cycle, since 76.3% of the dividing forms (Table I) occurred in this stage. Once the daughter cells reach the stage where they each possess their own nuclei the rate of growth is speeded up and only 16.1% of dividing forms are in this stage. Final separation of liberated daughter cells would appear to be the fastest process of all since only 7.6% of dividing forms were found in this stage at any one time.

We do not have sufficient information to interpret the significance of "anomalous" forms. Less than 1% of all cells seen were in the conditions shown in figures 23–28, 44, 45 compared to 14% showing the regular pattern of division. Possibly, aberrations were produced by the mechanical manipulations involved in preparing smears. Alternatively, it may be that the aberrations are regular occurrences, yielding viable or non-viable daughter cells. A third possibility is that if the daughter cells have some degree of mobility within the parent, they may have become fixed while in the process of moving around. The presence of these forms, however, does not alter the overall picture of reproduction by endodyogeny.

The question of how *Toxoplasma* manages to move in fluid media in the absence of visible flagella, cilia or other obvious means of locomotion, has vexed many investigators. We are able to repeat what most other observers have reported, that it was not possible to detect any locomotor organelles in living toxoplasmas. On the other hand, the presence of a distinct anterior spike similar to that shown in figure 27, was commonly seen in smears of formalin-washed organisms. Rarely on silver-stained smears, but frequently on smears stained with fluorescent antibody, a much longer, thread-like appendage was seen (Goldman, 1957). Westphal (1954) has demonstrated a delicate, anterior thread in living organisms by using electronic flash photographs taken with the phase microscope. The nature of this structure will be discussed in greater detail when a description of other organelles seen on silver-stained smears is reported, since the threads and spikes seems to be closely related to an internal rod-like structure which is present in *Toxoplasma* (Fig. 30).

Finally, it hardly seems necessary to emphasize how extremely valuable the silver protein method has been in the study of this organism. Not only has it enabled us to see practically all of the microscopic (as opposed to submicroscopic) structures revealed by electron microscopy, but the relationship of structures has been easier to understand than in thin sections of organisms. In view of the simplicity and availability of this technique compared to electron microscopy, it would seem to be the method of choice in studying organisms that may be related to *Toxoplasma*.

SUMMARY

Toxoplasma gondii from peritoneal exudate of infected mice has been studied in smears stained with silver protein. Evidence is presented that in such material this species reproduces by a process of internal budding which we are calling endodyogeny. In this process, each parent cell produces 2 daughter cells which develop internally. Upon reaching full growth, the joined daughter cells are freed of the parent cell, destroying the latter in the process. Final separation of daughter cells then takes place. Details of changes in the nucleus and associated cytoplasmic structures are described and figured.

ACKNOWLEDGMENTS

We are grateful to Dr. M. M. Brooke of the Laboratory Branch for conceiving and executing the text figure, and to Mrs. M. R. Borom of the Training Branch for the drawings on Plate II.

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EXPLANATION OF FIGURES

PLATE I

Figures 1 through 28 are photomicrographs taken with a 2-mm apochromat objective and a 25× eyepiece. The images were enlarged slightly and to varying degrees in printing, yielding final magnifications of about 3500×.

Figures 1–3. "Resting" or non-dividing stages.

FIGURE 1. Note position of the endosome and angular shape of the nucleoplasm. Fibrils extending posteriorly from the apical ring are clearly shown.

FIGURE 2. Shows 3 nuclear components: nucleoplasm, small nuclear granules, and large endosome. The apical ring is heavily stained but the anterior cone is not prominent.

FIGURE 3. Anterior portion of cell shows darkly stained anterior cone.

Figures 4–28. Dividing stages.

FIGURE 4. Organism at top shows very small nuclear buds, the earliest indication of reproduction. The anterior end is turned upwards showing the ring character of the anterior end of the cone.

FIGURE 5. Another early dividing stage showing direct sprouting from the parent nucleus. The anterior cone shows detail which is difficult to interpret at present.

FIGURE 6. Daughter cones are clearly developed but are still connected to parent nucleus.

FIGURE 7. Connection of daughter cones to parent nucleus cannot be seen. Parent endosome and nucleoplasm show no signs of division.

FIGURE 8. Dark-field photograph of stage similar to that shown in fig. 7. The apical rings of the parent and left daughter cell show perforations; the parent nucleoplasm appears as an amorphous cloudlike mass extending to the daughter cones; doughnut character of the endosome is suggested.

FIGURES 9, 10, 11. Developmental stages before daughter cells acquire separate nuclei.

FIGURE 12. Daughter cell on left shows a separate nucleus. The one on the right either does not have a nucleus yet or it is obscured. Note pointed anterior end of the organism in upper left corner.

FIGURE 13. Each daughter cell has its own nucleus which resembles the ordinary resting nucleus except for being smaller.

FIGURES 14, 15. Well developed daughter cells with large nuclear masses; relationship to parent nuclei is not clear because of dark stain. Note plate-like appearance of the anterior cone in fig. 14.

FIGURE 16. Dark-field photograph of stage similar to that shown in fig. 15. Perforation of parent and daughter anterior cones is clearly shown; also the 3 nuclear masses with endosome and nucleoplasm portions can be seen.

FIGURES 17, 18, 19. Development of daughter cells in anterior direction. Parent cell in fig. 18 is turned to reveal the apical ring; no parent nucleus is seen.

FIGURE 20. Daughter cells have broken out of the parent cell anteriorly but remain attached to the posterior portion which still contains the parent nucleus; note connections between all three nuclei.

FIGURES 21, 22. Daughter cells completely out of parent cell but still attached to each other.

FIGURES 23–28. Unusual and hard to interpret dividing stages.

PLATE II

FIGURES 29 through 45 are free-hand drawings made from smears showing much detail but stained too lightly to be photographed satisfactorily. Proper proportions and relationships were obtained by measuring each organism with a filar micrometer and drawing each to the same scale. Initial magnification was about 5000X, reduced by 10 per cent in printing.

Figures 29–35. "Resting" or non-dividing stages.

FIGURES 29–33. Various nuclear appearances are demonstrated. Notice the complete separation of endosome and nucleoplasm in figure 32. The lobed, doughnut character of the endosome is evident throughout. Figures 29 and 30 show the pointed anterior end of *Toxoplasma*. In 30 an internal rod-like structure appears to perforate the anterior end.

FIGURES 34, 35. The apical ring is clearly shown here.

Figures 36–45. Dividing Stages.

FIGURES 36, 37, 38. Early dividing forms. Parent nuclei show no evidence of division.

FIGURE 39. Daughter cells each have their own nucleus; parent nucleus with detached endosome is present posteriorly.

FIGURE 40. Daughter cells have grown anteriorly; the parent nucleus is not seen. The apical ring can be seen in each daughter cell.

FIGURE 41. Similar stage to figure 39; parent nucleus seems to lack endosome.

FIGURE 42. Daughter cells outside parent but still attached to each other along entire length of their bodies. Dotted lines at the anterior end have been put in to show the pointed end of each organism in order to clarify their relationships.

FIGURE 43. Paired organisms apparently pulling apart outside of parent cell.

FIGURE 44. Daughter cells developing at right angles to the undivided parent nucleus.

FIGURE 45. Well advanced daughter cells developing in the posterior direction; parent nucleus is not seen.

PLATE I

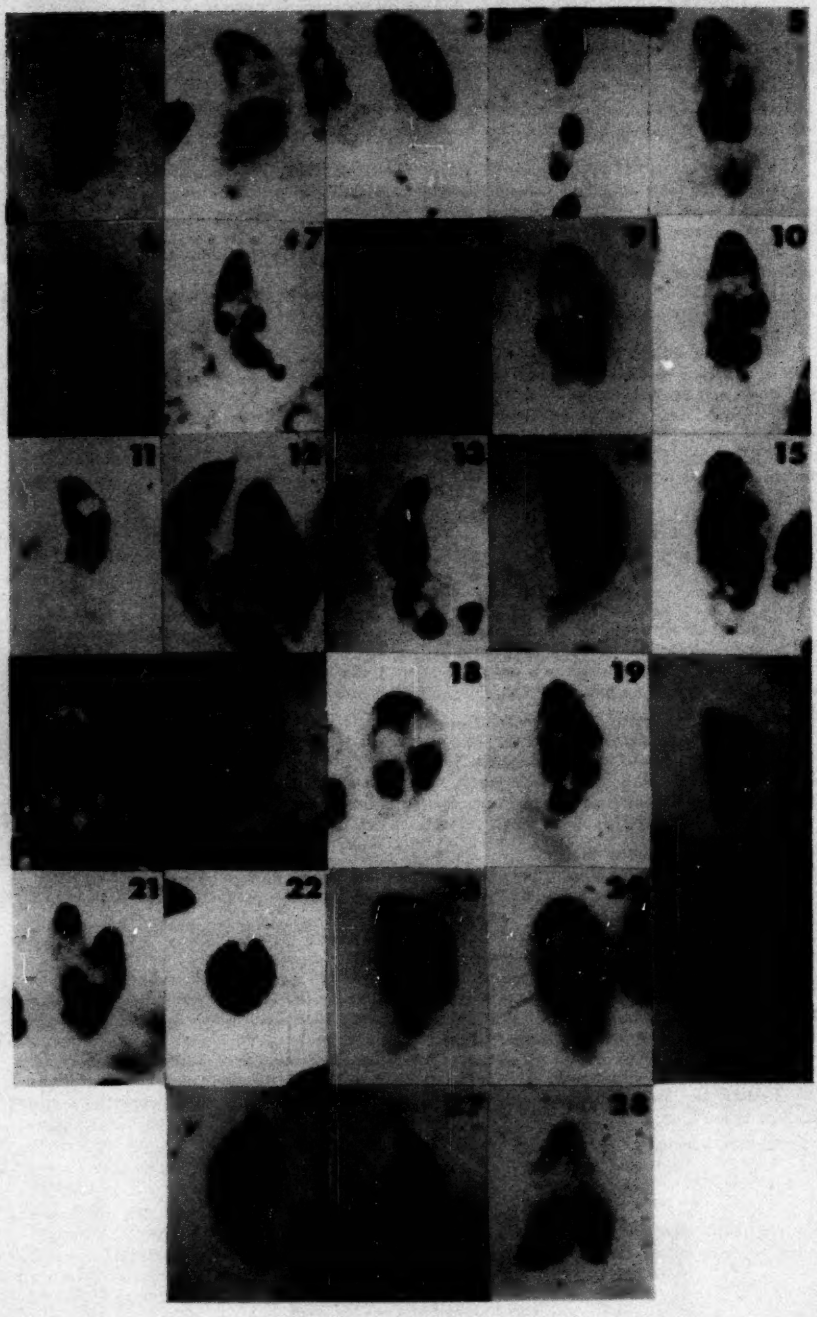
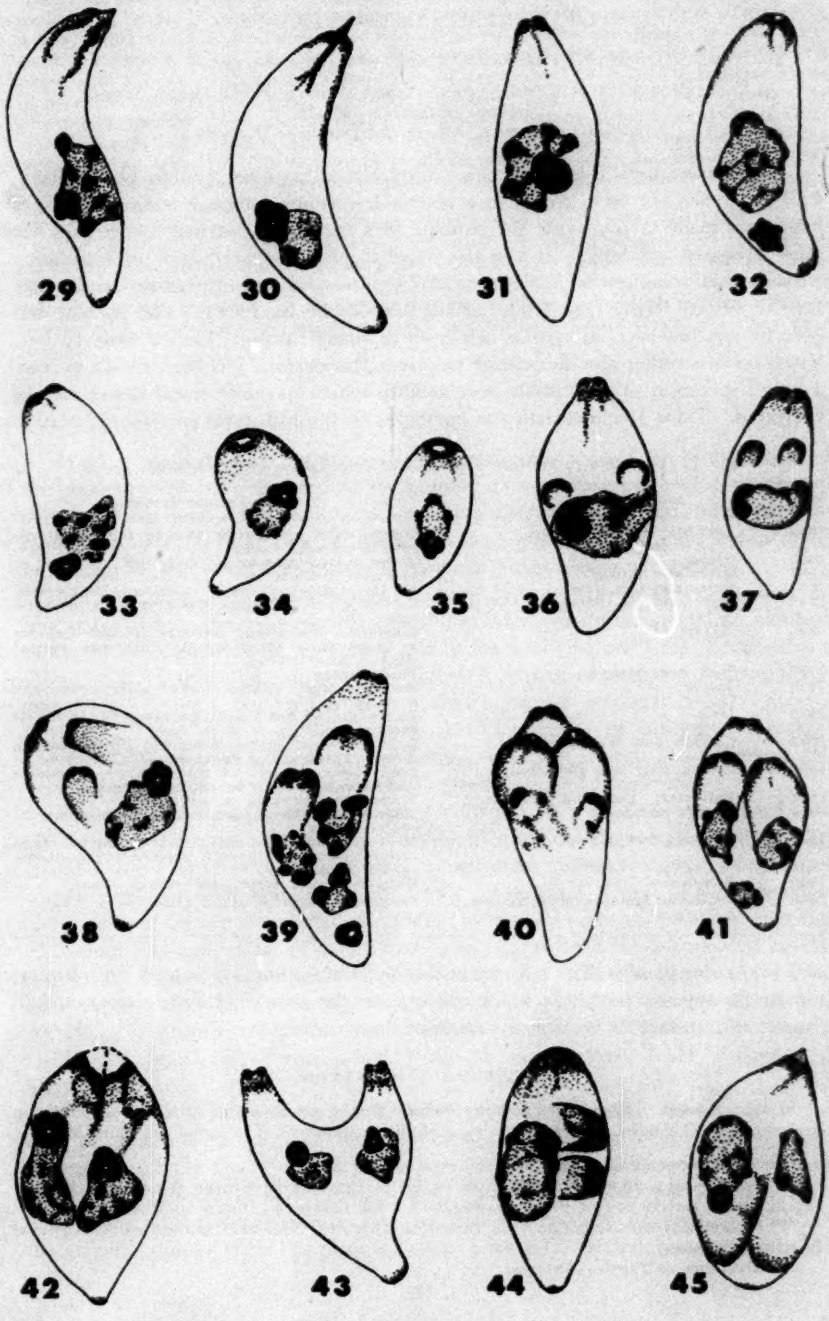


PLATE II



CULTIVATION OF *TOXOPLASMA GONDII* IN TISSUE CULTURES OF VARIOUS DERIVATIONS*

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Toxoplasma gondii is an obligate intracellular parasite (Sabin and Olitsky, 1937). It has not been grown in a nonviable medium although various attempts have been made to propagate the parasite in a variety of nutrient media used for other protozoa and fungi. It also has failed to grow extracellularly in association with *Trypanosoma cruzi* (Weinman, 1952) or to develop in the pooled embryonic fluid of 17-day fertile eggs (MacFarlane and Ruchman, 1948). The parasite has been grown, however, in various cell types in tissue culture. Unlike most viruses, which possess rather specific cellular tropisms, this parasite has been shown to have a broad spectrum of susceptible host cells in which its biochemical defect can be corrected. Table I summarizes the highlights in the history of *in vitro* cultivation

TABLE I. *Summary of the history of* in vitro *cultivation of* Toxoplasma gondii.

Date	*Investigators*	*Tissue Culture System*
1929	Levaditi, Sanchis-Bayarri, Lépine and Schoen	Minced chick embryos or chicken and pigeon tissues in chicken plasma.
1933	Bland	Tissue culture system not described.
1937	Sabin and Olitsky	Li-Rivers medium.
1942	Guimarães and Meyer	Maximow slide cultures of chick embryonic tissue.
1943	Meyer and de Oliveira	Cultivation in Maximow slides at room temperatures.
1952	Mühlpfordt	Embryonic muscle and cartilage of chick embryos in equal parts of embryonic juice and normal chicken blood plasma.
1953	Holz and Albrecht	Bone marrow.
1953	Jacobs	Maximow slide cultures of chick embryo liver and heart.
1953	Lock	Embryonic rat heart on flying coverslips in Carrel flasks.
1954	Chernin and Weller	Roller tubes of mouse embryonic tissues, human foreskin, uterus, and embryonic skin-muscle.
1954	Jacobs, Fair and Bickerton	Used tissue cultures of mouse mince to isolate *Toxoplasma* from a case of chorioretinitis.
1954	Pulvertaft, Valentine and Lane	Macrophages growing on serum-agar.
1954	Vischer and Suter	Macrophage cultures from immune and non-immune adult animals.
1956	Balducci and Tyrrell	Monolayer epithelial cell cultures derived from rabbit kidney and human epidermoid carcinoma (strain HeLa).
1956	Eichenwald	HeLa cells and trypsinized kidney tissue.
1956	Schuhová, Bonnové, Hübnera and Saskové	Stationary tubes of HeLa cells.

of *Toxoplasma gondii*. The purpose of this communication is to extend the information on the types of host cells which can support the growth of *Toxoplasma*, and to discuss certain factors in the cultivation of the parasite.

MATERIALS AND METHODS

Tissue cultures. Trypsinized monkey kidney tissues grown in roller tubes or on flying coverslips in roller tubes were used for most of the experiments. The initial medium (Melnick,

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1955) consisted of Hank's balanced salt solution at pH 6.8 containing 0.5% lactalbumin hydrolysate (Nutritional Biochemical Corp.), 2% calf serum (Microbiological Associates, Inc.) inactivated at 56° C for 35 minutes, with 100 units of penicillin and 100 micrograms of streptomycin per ml. After 4 or 5 days of initial growth at 37° C, the medium was changed to Earle's balanced salt solution at pH 7.6, containing 0.5% lactalbumin hydrolysate, 1% inactivated calf serum, with 100 units penicillin and 100 micrograms streptomycin per ml. A continuous monolayer of cells was usually formed at 4 to 7 days. Explant cultures of various other tissues were prepared by standard plasma clot techniques. Macrophage cultures were prepared by the technique of Carrel and Ebeling (1922). A variety of cultures was also purchased from Microbiological Associates, Inc. These cultures were maintained in Eagle's medium (Eagle, 1955) with 10% calf serum and the same concentrations of antibiotics stated above. Each tube received 1.5 ml of maintenance medium.

The mice were N. I. H. general purpose albino females weighing between 14–20 grams.

The RH strain of *Toxoplasma* was used and suspensions of the parasite were prepared by harvesting mouse peritoneal exudates into a 10% calf serum-Hank's solution. Organism counts were made in an AO Spencer Bright-Line haemacytometer and adjustment of the number of organisms present was made by appropriate dilution techniques using 10% calf-serum-Hank's solution as diluent. These inocula were always used within a 1-hour period after they were prepared.

Experimental

Types of Host Cells Parasitized

Multiplication of *Toxoplasma* and damage of cells caused by proliferation of the parasite have been observed by us in roller tube explant cultures of chick embryo heart, leg muscle, lung and intestine; in embryonic mouse heart, lung, leg muscle and intestine; in embryonic rat heart; in rabbit spleen, kidney and macrophages; in monkey spleen; in human foreskin, testicular tissue, and macrophages.

Multiplication of *Toxoplasma* and resulting damage to cells also have been observed by us in roller tube monolayer cultures of human amnion and of monkey, rabbit, guinea pig, beef and rat kidney and in the following cell lines: Strain HeLa (Gey), KB (Eagle), human intestine (Henle), human conjunctiva (Chang), human liver (Chang), human fibroblast (MAF), mouse sarcoma, S-180 (Foley), Earle's "L" line of mouse fibroblast, D-189 (Leighton) of malignant foreskin; Detroit-98 (Berman and Stulberg) of human marrow; Detroit-116 (Berman and Stulberg) of human pleura; and J-111 (Osgood) of human leukemia.

Monolayer cultures of normal monkey kidney cells (Fig. 1-A) inoculated with 200,000 RH strain toxoplasmas always showed complete degeneration (Fig. 1-D) on day 5 or 6 (day of infection = day 0). Inocula of 2 million toxoplasmas produced complete cellular degeneration on day 3 or 4. Figures 1-B to 1-D show progressive grades of cellular degeneration. Other monolayer cultures with the exception of conjunctival epithelium (Chang) and human intestine (Henle) showed similar degeneration times when infected with such inocula. The time required for complete degeneration of monolayer cultures of the various cell types inoculated with 200,000 RH strain toxoplasmas was as follows: KB, 7 to 8 days; S-180, 6 days; D-116, 6 days; D-189, 6 days; D-98, 5 days; HeLa, 6 days; J-111, 6 days; "L" fibroblast, 5 days.

When explant cultures, in which the outgrowth was mainly fibroblastic in nature, were inoculated with 2 million toxoplasmas, 4 days were required for complete degeneration. On day 4, diffuse fragmentation and death of the cells was evident.

In the conjunctival epithelium (Chang) and human intestine (Henle) there was considerable delay in cellular degeneration and cultures infected with 200,000 toxoplasmas did not show complete lysis even after a 12-day incubation period. Al-

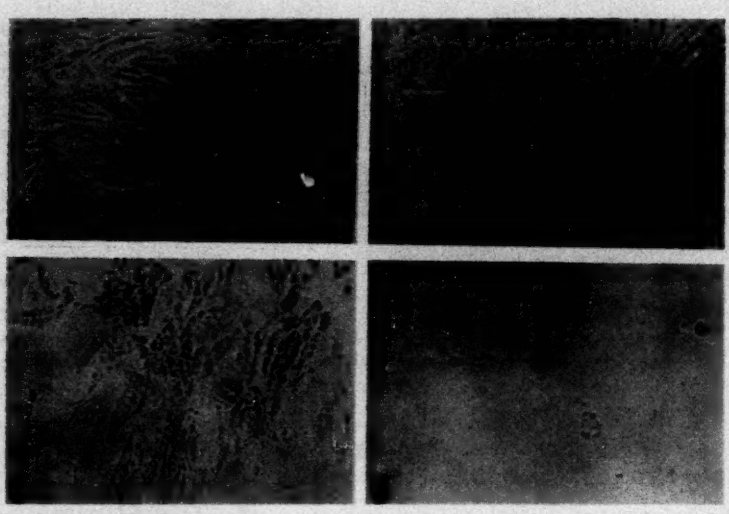

FIGURE 1. The effect of *Toxoplasma* on roller tube cultures of monkey kidney epithelium (×142).

A. Normal monkey kidney epithelial cells.
B. ± to + cellular degeneration.
C. 2+ cellular degeneration.
D. 4+ cellular degeneration.

though the epithelial cells were destroyed after about 9 days' incubation, and the cell sheath had lost its confluent nature, many fibroblast-like cells remained. These cells stayed in a healthy condition throughout 15 days' incubation, but mouse inoculations made from these cultures on day 15 were positive for *Toxoplasma*.

The infectivity of these cultures must be attributed to the presence of parasites within the surviving cells because studies, to be presented later, on the proliferative form of *Toxoplasma* show that the extracellular parasites survive only briefly in tissue culture medium.

Penetration

Another factor which was considered as possibly explanatory of the prolonged lysis times of the conjunctival and intestinal cell cultures as compared with monkey kidney epithelium was the time required for penetration of parasites into the various cells. This was tested in the following experiment: Stationary cultures of each of these cells were inoculated with 200,000 toxoplasmas. The organisms remaining

TABLE II. *Comparison of the numbers of RH strain toxoplasmas* remaining in supernatant fluids of 3 cell types after various incubation periods.*

Incubation Period (Hours)	Direct counts of 0.1 cubic millimeter of supernatant fluid from tissue cultures of . . .		
	Conjunctival Epithelium (Chang)	Human Intestine (Henle)	Monkey Kidney Epithelium
1	7.2×10^4	6.5×10^4	8.0×10^4
3	3.7×10^4	2.8×10^4	5.2×10^4
5	1.5×10^4	1.5×10^3	3.5×10^3

* 2×10^5 toxoplasmas were inoculated into each culture.

in the supernatant medium were counted at 1, 3, and 5 hours. Four counts were made in a haemacytometer from each of 5 or more cultures of each group. The results, presented in Table II, suggest that toxoplasmas gain entrance into conjunctival and intestinal cells as readily as into monkey kidney cells. Therefore, the prolonged lysis times of the first 2 types of cultures must be due either to some metabolic factors, to a physical difference such as the continuity of the cell sheets, or possibly to the resistance of the fibroblast-type cells which continued to proliferate.

Several experiments were also performed to determine the penetration time of *Toxoplasma* (RH strain) into monkey kidney cells in roller tubes and stationary cultures. The procedure was, in general, as follows: 200,000 toxoplasmas were inoculated into each of a series of roller tubes. At ½-hour intervals, groups of cultures were washed 3 times with maintenance medium and received 1.5 ml replacement medium. One half of the tubes in each group were reincubated and were examined daily for cellular degeneration while the remaining tissue cultures were pooled and inoculated intraperitoneally into mice (0.25 ml/mouse). Cultures from which the nutrient medium containing the extracellular parasites was removed at the end of ½ hour showed complete cellular degeneration in 6 to 8 days (average 6.8). At the end of 1 hour the same results were obtained. After 1.5 hours of exposure all the tissue cultures showed complete degeneration on day 6. The survival time of mice inoculated from all of these cultures was 6 days. Further decreases in lysis time of cultures and survival time in mice were not demonstrated in the later test periods up to 8 hours and at 24 hours.

Failure to demonstrate lysis time differences in stationary tubes versus roller tubes rules out the possibility that the inability of large numbers of the parasites to gain an intracellular position may be due simply to failure of the toxoplasmas to come in contact with the host cells for a sufficient time to allow active penetration or phagocytosis to occur. It is also possible that this phenomenon may be due to the presence of some non-suceptible cells in the cultures.

Several experiments were performed to test for loss of penetrative ability of toxoplasmas when the parasites were incubated in tissue cultures of monkey kidney epithelium at 37° C. In these experiments, cultures were inoculated with 200,000 toxoplasmas suspended in 1.0 ml of maintenance medium and incubated for one hour. At the end of this incubation period the supernatant medium was harvested from these tubes and pooled, and the cultures were re-fed with maintenance medium and re-incubated. Aliquots of the pooled supernatant harvested at the end of the first hour were used to inoculate clean tissue culture tubes. This procedure was repeated at the end of 2 hours.

When these cultures were read for cytopathic effect on day 5, the first group of cultures showed 4-plus cellular degeneration, while the second group showed 2-plus cytopathic effect. These data show that at least some of the parasites remaining in the supernatant medium at the end of the first 2 hours were able to invade other non-infected tissue cells in new cultures. Thus, there is an indication that the failure of the parasites to enter cells is not due to an inability on their part. The delay in cytopathic effect in the second group of cultures was probably due to a decrease in the number of viable organisms in the supernatant after incubation, as well as the loss of some parasites in the initial tissue culture tubes.

In another experiment tubes of maintenance medium without cells were inocu-

lated with 200,000 toxoplasmas/ml and were incubated at 37° C in roller drums. These tubes were sampled at 1, 5, 12, and 24 hours, and 0.25-ml aliquots, calculated to contain 10 toxoplasmas, were inoculated into each of 5 mice and 1.0 ml was inoculated into each of a series of tissue culture tubes. It was found on the basis of the mouse inoculations that beyond 5 hours there were few viable organisms remaining, and after 24 hours, the material was no longer infective. The mice inoculated from the tubes incubated for 1–5 hours were all positive for *Toxoplasma* but the tissue cultures inoculated with the incubated fluid showed only 1- to 2-plus lysis after 5 days' incubation as compared to 4-plus lysis on day 5 in tubes infected with the original inoculum prior to the 5-hour incubation period. The time increase required for complete cytopathic effect can be explained by loss of infectivity of some of the parasites after the 5-hour incubation period. The presence of cytopathic effect in these cultures indicates that the penetrative powers of the parasite in tissue cultures are retained as long as their infectivity for mice. These data suggest, therefore, that failures to obtain clearing of the supernatant of parasites in the experiments described earlier may be due to the initial non-susceptibility of some of the cells in the tissue culture system.

Pulvertaft *et al* (1954) studied, by lapse-time phase photomicrography, various types of fibroblastic, lymphocytic, and macrophage-type cells growing on serum-agar. They gave no information on the time required for penetration of parasites into cells but reported that active invasion by *Toxoplasma* was lethal to the cells and that reproduction of the parasite was confined to those which were phagocytized. It is difficult to assess the role of phagocytosis of the parasites by monkey kidney epithelial cells. Shepard (1955) demonstrated that these and HeLa cells will phagocytize human tubercle bacilli and Dr. C. P. Li (personal communication) has shown that monkey kidney cells phagocytize amoebic cysts in tissue culture. However, the hypothesis that active invasion by the toxoplasmas consistently results in death of the host cell is not supported by the studies reported here. In the microscopic examinations of parasitized cultures, the outgrowths from explants and the monolayer sheaths showed no evidence of cell loss early after infection of the cultures. It is possible that under the special conditions of cultivation on serum-agar, injury to the cell membrane due to invasion caused a damage not occurring in roller tube or coverslip preparations.

Effects of Culture Modifications

Differences in lysis times possibly could be dependent on varied metabolic rates of different cell cultures. While it was not feasible to test this accurately by metabolic studies on various cell lines, it was possible to observe differences in the proliferation of toxoplasmas in 1 cell line under varied conditions. The following experiments were performed with this objective.

Tissue cultures infected with 200,000 toxoplasmas were maintained at 35° C, 22° C, and 4° C. At 35° C there is active cellular growth and metabolism, and complete cellular degeneration due to proliferation of toxoplasmas was noted on day 6. At 22° C, which is a temperature low enough to prevent or reduce cell division to a minimum and to retard cell respiration (Hecke, 1930; Sanders, 1940), complete cellular degeneration due to proliferation of toxoplasmas was not apparent until the 28th day of infection. The cultures maintained at 4° C showed no degeneration due to proliferation of toxoplasmas after 30 days and these cultures then were noninfective to mice by intraperitoneal inoculation.

Two variables exist when cultures infected with *Toxoplasma* are exposed to lowered temperatures. The above results indicate that there is a direct relation between temperature and the multiplication of *Toxoplasma* and also suggest that, through a complex interaction of the 2 variables in the system, there may be a relation between the lowered rate of metabolism of the host cells and the lowered rate of multiplication of the parasite. In addition, the results indicate that the parasites may multiply within a fairly wide range of temperatures. The decreased rate of multiplication of toxoplasmas in tissue cultures maintained at 22° C is reminiscent of the findings of Rodhain (1951) who reported the prolonged course of a *Toxoplasma* infection in a hibernating marmot. It is of interest also that Meyer and de Oliveira (1943) and we have used tissue cultures incubated at room temperature to maintain strains of *Toxoplasma*. Incubation at this temperature eliminates the frequent medium changes and transfers necessary when cultures are incubated at 37° C. In this laboratory, strains of *Toxoplasma* have been maintained in tissue cultures of monkey kidney epithelium incubated at room temperature for a period of 3 months through 3 serial passages.

The effect of biological activity of the host cell on the multiplication of *Toxoplasma* was also investigated by the use of colchicine. This was stimulated by the report of Bozerman *et al* (1956) that rickettsiae continued to multiply in colchicine-arrested MB-III cells.

In the experiments reported here 0.1 gamma/ml of colchicine was added to tubes of monkey kidney cells 96 hours prior to infection with *Toxoplasma*, and this concentration was maintained throughout the experiment. Non-drugged cultures were employed as controls. An inoculum of 200,000 toxoplasmas was added to each tube.

Because in the presence of colchicine the sheaths of cells were greatly disrupted, it was necessary to use criteria other than lysis time to measure the extent of proliferation of *Toxoplasma*. Titrations of infectivity for mice of treated and untreated cultures were performed every 24 hours for 5 days. A summary of these titrations is shown in Table III. First, it appears that toxoplasmas were able to penetrate

TABLE III. *Comparison of the growth of toxoplasmas in colchicine[1]-treated and non-treated cultures of monkey kidney epithelium.*

Hours	Non-Treated			Treated		
	Highest infective dilution	No. of mice dead of 5 inoculated	Survival times	Highest infective dilution	No. of mice dead of 5 inoculated	Survival times
24	10^{-3}	3	10, 10, 10	10^{-3}	4	8, 9, 9, 10
48	10^{-5}	3	9, 9, 12	10^{-4}	3	9, 11, 12
72	10^{-5*}	2	9, 10	10^{-7*}	4	9, 9, 9, 11
96	10^{-5*}	2	9, 9	10^{-4*}	3	9, 9, 9
120	10^{-7}	5	7, 8, 8, 8, 8	10^{-4*}	3	8, 8, 8

[1] The concentration of colchicine used was 0.1 gamma/ml.
* Highest dilution tested.

treated and untreated cells equally well. Secondly, while it was not true at all intervals at which tests were run, nevertheless the infectivity titers of colchicine-treated cultures at 24 and 72 hours indicate that proliferation had proceeded as rapidly in the presence of colchicine as in its absence.

Additional data derived from microscopic observation showed that by the third day of infection colchicine-treated cells had assumed great size and many unusual shapes. Relatively few remained adherent to the walls of the tubes. These en-

larged and distorted cells harbored more parasites than the non-treated cells. While the non-treated cells were showing 1- to 2-plus lysis by the fifth day of infection, there was no evidence of degeneration due to *Toxoplasma* in any of the colchicine-arrested cells. The ability of the colchicine-treated cells to harbor more parasites may be related to the availability of cytoplasm present in the arrested cells. The growth of *Toxoplasma* within them is indicative of the ability of this parasite to proliferate even though there is interference with some biological activity of the host cell.

Experiments were also performed to determine the effect of age of the tissue cultures on multiplication of *Toxoplasma*. Cultures of monkey kidney epithelium were inoculated with 200,000 toxoplasmas 6, 14, 21, and 28 days after the cultures were initiated. Cellular degeneration was complete by day 6 in each group and no variation in the lysis times of the cells due to proliferating toxoplasmas was noted between the age groups.

Cultures maintained without antibiotics showed no differences in lysis times following infection with *Toxoplasma*.

Virulence

Sabin and Olitsky (1937) reported that 2 series of 6 subcultures of toxoplasmas in minced chick embryo suspended in Tyrode's solution did not result in a loss of pathogenicity of the parasite. Meyer and de Oliveira (1945) passaged *T. gondii* in tissue cultures for long periods without detecting loss of virulence for mice. Chernin and Weller (1954) cultivated the RH strain of *Toxoplasma* in roller tubes of mouse embryonic tissues through 15 serial passages without an apparent loss in virulence of the organism.

Schuhová *et al* (1956) grew strain "CB" of *T. gondii* in stationary tube cultures of HeLa cells and reported that "the number of extracellular parasites increased in subsequent passages due to adaptation of the parasite to the host cell." However, in a later paper, the same senior author states that infected cultures of HeLa cells could be maintained as long as 6 months in medium containing 40% horse serum in Hank's solution, by changing the medium every 3–4 days. Since many horse serums contain an anti-*Toxoplasma* factor (Sabin and Feldman, 1948) it may be that this prolonged delay in lysis was due to such an inhibiting influence. Because up to now we have seen only abstracts of these papers, without details, it is not possible to judge whether or not a change in virulence of the parasite could be considered as an explanation for the findings.

In an attempt to study the possibility of the occurrence of a modification in virulence in *Toxoplasma*, a strain of the parasite derived by isolation in mice from a single patient (LB) was thereafter maintained both in mice (LB-IP) and in tissue cultures (LB-TC) by serial passages in tubes of monkey kidney epithelial cells for a period of 10 months through 43 serial passages.

A tissue culture control series was also maintained. Fluids from each lot of cells used to maintain the strains of *Toxoplasma* were serially passed to rule out the presence of contamination with a "wild agent" derived from the cells themselves; no such agent was demonstrated.

After 43 passages in tissue cultures and 103 passages in mice, differences in virulence of the 2 lines were measured on the basis of survival time of mice inoculated with logarithmic dilutions of tissue culture fluids or peritoneal exudates contain-

ing toxoplasmas. Five mice were inoculated intraperitoneally from each dilution.

When the 50% mortality dose for mice was calculated from the data in Table IV by the method of Reed and Muench (1938), it was found that the LD_{50} for the

TABLE IV. *Comparison of the virulence of 2 lines of strain LB of* T. gondii.

Dilution of Inoculum	Tissue Culture Line (LB-TC)			Mouse Line (LB-IP)		
	Number of mice dead*	Survival time (days)	Average survival time	Number of mice dead*	Survival time (days)	Average survival time
10^{-2}				1	8	8.0
10^{-3}				4	7, 8, 8, 8	7.75
10^{-4}	0	—	—	5	7, 7, 7, 7, 8	7.2
10^{-5}	1	10	10	5	6, 7, 7, 8, 8	7.2
10^{-6}	4	10, 10, 11, 11	10.5	4	7, 8, 8, 8	7.75
10^{-7}	5	9, 9, 10, 10, 11	9.8	5	8, 8, 8, 8, 8	8.0
10^{-8}	5	8, 9, 9, 9, 10	9.0	5	7, 7, 7, 7, 7	7.0
10^{-9}	5	7, 7, 7, 7, 7	7.0	5	5, 5, 5, 5, 6	5.2
10^{-10}	5	6, 7, 7, 7, 8	7.0	5	4, 5, 5, 5, 5	4.8
10^{-11}	5	6, 6, 6, 6, 9	6.6			

* 5 mice were inoculated in each group.

LB-TC line was between 10^{-5} and 10^{-6}, while the LD_{50} for the LB-IP line was between 10^{-8} and 10^{-9}. Inspection of the table reveals that there is approximately a 2-day difference in the survival times of mice inoculated with these 2 lines of the parasite when compared at the LD_{50}.

Survival time studies were also performed in hamsters. Five hamsters were each inoculated intraperitoneally with 1,000 LB-IP or LB-TC toxoplasmas. In the 5 hamsters inoculated with LB-TC toxoplasmas, the survival days were 7, 8, 8, 8, 9 (average 8.0 days). The 5 hamsters inoculated with LB-IP toxoplasmas died in 5, 6, 7, 7, and 8 days (average 6.6 days).

While these data appear to show a lower virulence for the LB-TC line as compared to LB-IP parasites, it is not justifiable to consider this an alteration of the parasites passed in tissue culture. In addition to this possibility, the mouse-passed line may have been enhanced in virulence. Continued passage in mice has been shown to result in increased virulence as compared with the same strain maintained in chick embryos (Jacobs and Melton, 1954). Unfortunately a chick embryo-passed line which was being maintained for comparison with LB-IP was lost accidentally before the tests could be made. It is considered likely, however, that no alteration in virulence of LB-TC occurred, but that, rather, as suggested by Jacobs (1956) tissue cultures may be useful in maintaining strains at their original level of virulence.

Failure To Demonstrate Toxin Production

The report of "toxotoxin" by Weinman and Klatchko (1950) prompted an experiment to determine the existence of such a substance in the infected tissue cultures and its possible role in cellular degeneration.

Fluids from tissue cultures showing 2- to 4-plus degeneration were harvested, pooled, and were centrifuged at 2,500 rpm for 20 minutes in a Number One International Centrifuge. The sediment containing tissue culture cells, toxoplasmas, and debris was discarded and aliquots of the supernatant were treated by (1) heating at 45° C in a water bath for 30 minutes; (2) alternately freezing and thawing 7 times in a 95% alcohol-dry ice bath and a 22° C water bath; (3) storing at 4° C for 48 hours; (4) centrifuging in a Spinco centrifuge at 4° C at 15,000 rpm for 30 minutes; and (5) filtering through a Selas (#02 porosity) candle. The control

for this experiment was tissue culture media containing viable toxoplasmas harvested from infected tissue cultures showing 2- to 4-plus cellular degeneration. One-half ml of each of the above fluids was inoculated into each of 5 tissue culture tubes of monkey kidney epithelium. The only fluid producing any cytopathic effect was the control fluid containing viable toxoplasmas. There was no evidence of the presence of a toxin in any of these experiments or in any of the other tissue culture experiments performed in this investigation. These results confirm the findings of Chernin and Weller (1954), Balducci and Tyrrell (1956) and Varela, Roch and Vazquez (1955) who also failed to demonstrate toxin production by *T. gondii*.

DISCUSSION

It is obvious that *Toxoplasma gondii* is very indiscriminate as to the host cells it will invade and within which it will grow. There are some indications that the proliferation of *Toxoplasma* may be dependent on the rate of host cell metabolism; this would suggest dependence on some metabolite produced within the host cell. The studies on colchicine-treated cells indicate that this dependency is not related to those activities involving mitotic division which are disturbed by colchicine.

The factor(s) necessary to correct whatever biochemical defect *Toxoplasma* possesses is (are) obviously present in a wide variety of cells. This suggests that it may be possible to define the conditions for extracellular growth of this parasite. Biochemical studies on this parasite would be profitable, such as those on rickettsiae by Bovarnick and Allen (1954) who showed the loss by elution of the coenzymes DPN and A from *R. prowazeki*, and those of Consigli *et al* (1957) who prepared a complex medium for extracellular growth of *R. akari*. The recent report of Fulton and Spooner (1957) who prepared pure extracellular suspensions of *Toxoplasma* by filtration to remove host cells, and who then performed preliminary metabolic studies on the parasite, provides some means for such investigations. In addition, standardized host cell suspensions, arrested or altered at some stage in their metabolism, as by colchicine, may be useful for these purposes.

Tissue cultures may also provide means for *in vitro* studies on immune mechanisms involved in toxoplasmosis. The evidence presented above, that the tissue cultures used here apparently contained both susceptible and non-susceptible cells, suggests that it would be necessary to use clones derived from susceptible cells for such studies.

SUMMARY

1. A broad spectrum of mammalian and avian cells which will support the growth of *Toxoplasma gondii* has been reported.

2. Evidence has been presented which suggests the presence of non-susceptible cells in cultures of monkey kidney tissue.

3. A relation between temperature and the multiplication of *Toxoplasma* has been shown in tissue cultures.

4. Colchicine-arrested cultures of monkey cells apparently are equally as able to support the growth of *Toxoplasma* as non-treated cells.

5. No variation in the lysis times of cells in tissue cultures due to the proliferation of toxoplasmas was noted between cells of various age groups.

6. The degree of virulence of a strain of *Toxoplasma* appeared to be maintained after prolonged serial passage in tissue cultures.

7. Toxin production by toxoplasmas growing in tissue cultures was not shown.

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RESEARCH NOTE

SOME PARASITES OF THE WESTERN SPOTTED FROG, *RANA P. PRETIOSA*, IN YELLOWSTONE PARK, WYOMING*

Little is known of the helminth parasites of *Rana p. pretiosa*. Ingles (1936, Trans. Amer. Micr. Soc. **55**: 73–92) reported *Spironoura pretiosa* and *Gorgoderina multilobata* from *Rana pretiosa* in Plumas County, California, but these frogs were almost certainly what is now considered *Rana cascadae*. Lucker (1931, Proc. U. S. Nat. Mus. **79**, Art. **19**: 1–8) described *Haplometrana intestinalis* from the intestine of *Rana pretiosa* from Bothell, Washington. Olsen has described *Haplometrana utahensis* and *Gorgoderina tanneri* (1937, J. Parasitol. **23**: 13–25 and 499–503) and the nematode *Aplectana gigantica* (1938, Trans. Amer. Micr. Soc. **57**: 200–203) from the western spotted frog from Springville, Utah. Schonberger (1945, Copeia **2**: 120–121) mentions that in a collection of 37 spotted frogs only 2 exhibited "ascarid parasitism."

The examination of gut contents of *Rana p. pretiosa* collected from populations near Fishing Bridge in Yellowstone National Park, Wyoming, during 1954 and 1955, permitted incidental observations of helminth parasites of this anuran.

Of 62 hosts examined 32 were parasitized by the nematode *Spironoura pretiosa*, 20 by lung flukes (*Haematoloechus similiplexus* and another undetermined species of this genus), and 10 by an undetermined species of *Glypthelmins*. *Gorgoderina tanneri* infected 8 of 27 frogs examined and an undetermined species of *Halipegus* was found in 4 of 46 hosts inspected. No cestodes were observed.

A comparison of the parasitized frogs with the entire sample revealed no differences in the infection of males and females. However, the heaviest observed infections of all parasites occurred in large adult hosts. If only 2 host size-groups (20 to 50 mm and 51 + mm) are considered, Chi-square analyses indicate that the apparently higher rate of infection of large frogs is statistically significant (except in the case of *Halipegus*). The probability of accepting this conclusion erroneously ranges from about 0.06 in the case of *Glypthelmins* to less than 0.01 in the cases of *Spironoura* and *Haematoloechus*. These data would seem to reflect the fact that larger (older) frogs have simply been exposed to the possibility of infection for longer periods of time than smaller ones. Differences in the amount of food ingested as well as qualitative differences in the diet of large and small frogs may also be involved.

While it may be affirmed that *Haematoloechus* and *Spironoura* occur commonly in every population investigated the small samples involved preclude any definite assertion as to whether some of the populations sampled may be free of certain of the other parasites.

The writer wishes gratefully to acknowledge the aid of Dr. Marietta Voge and Dr. E. K. Markell who, as the condition of the material permitted, identified the trematodes; and of Dr. A. C. Walton, who identified the nematodes. The cooperation of members of the United States National Park Service in Yellowstone Park, Wyoming, the Museum of Vertebrate Zoology, and the Department of Zoology, University of California, is also acknowledged.—FREDERICK B. TURNER, *Department of Biology, Wayne State University, Detroit 2, Michigan and Museum of Vertebrate Zoology, University of California, Berkeley.* *(From a dissertation, in part, submitted at the University of California, Berkeley, under the supervision of Robert C. Stebbins.)

EFFECTS OF NUMBERS OF *ASCARIDIA GALLI* IN DEPRESSING WEIGHT GAINS IN CHICKS[1]

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Helminthologists have generally assumed that host damage by a given species of parasite is dependent, in part, upon the number of worms present with severity of damage increasing with the number of worms. As far back as 1922 Darling indicated the desirability of determining the number of hookworms present in infected individuals. As this work was confirmed and elaborated upon by others, clinicians came gradually to consider hookworm infections of less than 50 worms as non-clinical cases. Similar generalizations have also been proposed for other types of helminths. A high incidence of parasitism in poultry has been demonstrated with the large intestinal roundworm, *Ascaridia galli*, and vast sums are being expended on de-worming compounds. It seemed desirable to determine whether there might be a similar subclinical level of parasitism with this species. However, Todd and Hansen (1951) reached an opposite conclusion regarding the reliability of worm number as an index of degree of clinical parasitism in this species. In a series of experiments on 566 2-week-old chicks each of which had been exposed to about 50 eggs of *A. galli*, they found when birds were killed 3 weeks later that hosts with no worms gained less than those with 1 worm. A progression of weight gains was found which was correlated with increasing numbers of worms up to 15 per bird. From these data the authors developed a thesis that energy employed by animals resisting infection prevented maximum efficiency in gaining weight. Since these experimental results are contrary to the generally accepted view of parasitism, this new theory must either be substantiated or denied before a satisfactory yardstick can be established to determine effects of parasitism using worm numbers as a criterion of clinical parasitosis. Thus a series of experiments was set up to see if this new theory could be substantiated.

MATERIALS AND METHODS

Broiler strains of White Rocks were obtained from local hatcheries as day-old chicks. During the first week, vaccination for Newcastle disease was carried out by administering the B_1 strain in the drinking water. Birds were maintained on commercial starting or growing feeds. At 3 weeks of age, birds were weighed, paired with a control used in other studies, and parasitized with 300 larvated eggs of *A. galli*. Weekly weights were obtained until birds were sacrificed 3 weeks later. *A. galli* eggs were cultured and worms were collected and measured by a combination of methods reviewed by Reid, Pate and Kleckner (1957).

Since this study was designed to compare with the work of Todd and Hansen certain differences in technique should be noted. Three hundred in place of 50 eggs were fed in an attempt to get a heavier worm burden. Todd and Hansen had indicated a paucity of data on more heavily infected birds. Eggs were fed to 3-week-old chicks rather than at 2 weeks in order that the data on weight gains could be used in other studies. It was impossible to duplicate the high degree of genetic homogeneity of chicks used by these other investigators. The 5 separate experiments were carried out on chicks raised from different flocks.

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Within experiments, linear and curvilinear regression analyses were conducted in order to remove the average effects of individual experiments. This statistical treatment makes the reality of results for weight comparisons similar to those of Todd and Hansen. For the curved regression a second degree polynomial was fitted to the data. Since most of the birds contained 10 or less worms this part of the data was first analyzed. The second analysis included all of the birds.

RESULTS

Five different experiments were performed on a total of 338 control and 338 parasitized chicks which contained a total of 1,957 worms. Some of these data have been reported in connection with an infectious bronchitis study (Reid, Pate, and Kleckner, 1957). Parasitized birds, killed 3 weeks after infection, gained less in 4 out of 5 experiments than the unparasitized controls. Combined data showed significantly less weight gain for parasitized birds at a 90% level of probability. The number of parasitized birds in the 5 experiments varied as follows: Experiment 1, 45; 2, 44; 3, 63; 4, 88; and 5, 98 birds. Effects of variability were removed before the regression values were calculated. Variability was due (1) to differences between experiments including sex differences, males having been used in Experiments 3 and 4 and mixed sexes in the others, and (2) to variation within Experiments 4 and 5 where one group of birds was exposed to infectious bronchitis while the other group was not. The combined data are included in summary form in Table I to give some idea of the range and variability obtained in this study.

TABLE I. *Weight gains of chicks 3 weeks after parasitizing together with worm measurements in order of the number of worms recovered.*

Experiment No.	No. worms recovered	No. Birds	Range of weight gains in grams	Mean gain in grams	Range of worm length in mm.	Mean worm length in mm.
1	0 (unparasi-	43	364–713	482	———	—
2	0 tized con-	45	153–474	325	———	—
3	0 trols)	63	165–584	436	———	—
4	0	87	153–656	502	———	—
5	0	100	267–554	415	———	—
1	0 (parasitized)	9	449–561	495	———	—
2	0	13	200–412	325	———	—
3	0	6	270–550	417	———	—
4	0	11	288–575	455	———	—
5	0	19	202–482	372	———	—
1	1	6	355–562	479	5.2– 18.0	10.5
2	1	5	294–412	375	3.3– 28.6	13.8
3	1	13	150–508	381	2.2– 15.0	6.6
4	1	23	– 29–619	474	1.6– 28.0	14.7
5	1	13	317–530	411	1.9– 5.0	3.7
1	2	3	360–633	519	4.0– 12.9	9.1
3	2	7	277–501	389	2.0– 20.0	9.1
4	2	15	386–575	493	6.0– 31.0	15.7
5	2	19	295–554	412	1.8– 16.0	5.0
1	3	2	394–463	429	16.0– 19.6	17.8
2	3	4	246–406	353	2.5– 24.0	8.2
3	3	6	238–468	361	2.1– 24.0	7.3
4	3	11	448–645	523	2.0– 32.0	18.3
5	3	15	279–544	424	1.1– 15.0	4.9
1	4	2	410–454	432	2.3– 12.0	7.5
2	4	4	300–389	349	2.0– 14.6	6.7
3	4	13	116–534	375	.6– 28.0	6.7
4	4	13	383–533	488	2.0– 32.0	18.1
5	4	3	330–465	416	1.9– 23.0	6.0
1	5	2	426–492	459	3.0– 8.0	5.7
3	5	5	384–479	444	.6– 33.0	10.8
4	5	6	297–606	462	6.0– 32.0	17.1
5	5	4	322–455	382	2.7– 15.1	6.8
1	6	4	395–458	435	2.0– 23.0	12.3
2	6	1	386–386	386	2.2– 25.0	13.0
3	6	3	202–303	256	1.8– 20.0	6.6
4	6	5	486–568	526	3.0– 34.0	15.5
5	6	4	275–524	398	2.5– 23.0	8.9
2–5	7	11	315–502	405	1.0– 6.9	8.8
1–5	8	13	240–517	360	2.0– 93.2	10.3
1, 3, 5	9–10	6	338–461	379	1.1– 26.0	7.0
1–5	11–19	23	86–522	392	1.0– 67.6	11.3
1–3	23–29	6	310–651	426	1.1–123.6	12.2
1–3	33–48	4	297–399	362	2.0– 34.0	13.7
1–3, 5	52–98	6	178–477	332	1.0– 45.0	14.3

Of the 338 parasitized birds, 58 contained no worms, and the majority contained less than 10. The weight gain of the birds with no worms ranged from 200 to 590 grams, with an average of 401 grams. The range in weight gain for birds with 1 worm was −29 to 593 grams giving a mean weight gain of 433 grams. Similar variation among the birds is evident throughout the 5 experiments.

The regression coefficient (b) of weight gain on worm number for birds with 10 worms or less was negative ($b = -2.86g \pm 1.63$) and non-significant. The correlation coefficient was −0.10 with 290 degrees of freedom.

When the data for the 338 birds were analyzed, a highly significant negative linear regression ($b = -1.39g \pm 0.37$) was obtained. Thus each worm may be considered as causing a weight loss of 1.39 grams during the 3-week period of these experiments. This figure may prove useful in assessing economic losses produced by this parasite. The correlation coefficient was −0.20. The predicted weight gain can be obtained from the following equation: $\hat{y} = 425.30 - 1.39X$ where $\hat{y}$ equals predicted weight gain and X equals the number of worms. From observing the plot of the data, it would appear that the relation of weight gains on worm number was not linear; therefore, a second degree polynomial was fitted to the data. It was found that the curved regression fitted the data significantly better than the linear regression. The multiple correlation coefficient of 0.26 was highly significant.

Predicated weight gains based on the curved regression were calculated by the use of the following equation: $\hat{y} = 438.073 - 4.946X + 0.046X^2$ where $\hat{y}$ equals predicted weight gain and X equals the number of worms. This curve indicated that as the number of worms increased, weight gains were slower. This relation was almost a straight line with 40 or less worms. After this point, an increase in worm load did not affect weight gains to any great extent.

A highly significant positive regression coefficient of worm length on worm number was obtained ($b = 0.168mm \pm 0.03$). The correlation coefficient was 0.88.

DISCUSSION

From their data, Todd and Hansen (1951) concluded that birds exposed to a uniform parasitic infection weighed less if they had fewer worms than if they had many. From these data they developed a thesis that birds which expended energy in throwing off infection gained less weight than the ones which tolerated infection. Although this thesis is again mentioned by Egerton (1953) and Egerton and Hansen (1955) the latter papers do not present additional substantiating data. The data presented in the present work do not substantiate the findings of Todd and Hansen in regard to weight gain. The present report supports the opposite conclusion—the more worms present, the more the weight gain of the bird is depressed. Careful study of the differences in experimental procedure does not show any apparent reason for different results. The other investigators apparently severed the intestine at the yolk sac diverticulum which results in missing some of the smaller worms in the lower intestine (Edgar, Davis and Frazier, 1957). This possible source of error would not appear to answer the question as to why results from their series of experiments differ from ours.

The findings in the present experiment would substantiate the classic theory that worm damage is directly dependent upon worm number rather than the thesis

of Todd and Hansen. Results in the present experiments agreed with their report on worm length. The progression of increased worm lengths was correlated with increased numbers of worms found.

SUMMARY

1. In an experiment involving 338 chicks exposed to 300 infective eggs of *Ascaridia galli* it was found that birds had a highly significant decrease in weight gain as the number of worms increased. Birds with no or few worms had gained more during the 3 weeks after infection than had birds with larger numbers. These data appear to fit a second-degree polynomial regression curve. From statistical calculations each worm in these experiments may be considered responsible for a weight loss of 1.39 ± 0.37 grams. This figure may be useful in appraising economic losses.

2. These findings support the classic idea of helminthologists that worm damage, in part, is dependent upon the number of worms present; they do not support the thesis that chickens harboring few *A. galli* gain less weight than those harboring many.

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RESEARCH NOTE

COOPERIA SPATULATA RECOVERED FROM CATTLE IN THE UNITED STATES

In the course of a survey to determine the prevalence of worm parasites in cattle in southern Georgia, the writer recovered from 2 of the animals examined a total of 480 *Cooperia* males, which he has identified as *C. spatulata* Baylis, 1938. So far as the writer can ascertain, this species has not previously been reported to occur in the United States or outside of Australia and the Federation of Malaya. In making the identification, 29 of the specimens were carefully examined. They were 6.2 to 8.2 mm in length, and their spicules measured 224 to 270 microns. Baylis (1938, Ann. and Mag. Nat. Hist. **1**: 68–73) described the males of *C. spatulata* as being 6.3 to 7.5 mm long, with spicules measuring 230 to 290 microns. Through the courtesy of the British Museum, Mr. John T. Lucker, of the Animal Disease and Parasite Research Division, Beltsville, Maryland, was able to borrow and to compare 3 male specimens, which were identified by Baylis as *C. spatulata*, with 5 male specimens, tentatively identified by the writer as *C. spatulata* from Georgia cattle. Mr. Lucker considered the 2 lots of specimens to be conspecific, thus confirming the presence of *C. spatulata* in cattle in the United States.—WILLARD W. BECKLUND, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Tifton, Georgia.*

IMMUNITY TO CHALLENGING INFECTIONS OF *LITOMOSOIDES CARINII* PRODUCED BY TRANSFER OF DEVELOPING WORMS[1]

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Previous experiments with *Litomosoides carinii,* the filarial worm of the cotton rat, have shown that worms of an infection produced by subcutaneous introduction of infective larvae are retarded in growth and development when the infection is superimposed on an existing infection produced by the same means (Macdonald and Scott, 1953). Other experiments have shown that when the primary infection is produced by surgical transfer of fourth-stage larvae or young adult worms, the retardation of growth of a subsequent infection produced by subcutaneous introduction of infective larvae is very much less than in the previous case (Scott *et al,* 1956). The present experiments were undertaken to determine the effect of primary infections produced by transfer of worms in an intermediate growth stage, namely those in the late third larval stage. A preliminary account of these experiments has been presented by Scott and Macdonald (1956).

For purposes of discussion, we are calling this retardation of growth and development an expression of "immunity." In this paper the words immune and immunize will be used only in this sense.

METHODS AND MATERIALS

Infective larvae of *Litomosoides carinii* were introduced subcutaneously into each of a series of previously uninfected donor cotton rats by the cover glass method described by Scott and Macdonald (1953). Each rat was killed 7 days after infection, and the developing worms were washed from the pleural cavity in saline solution. The worms were placed on a cover glass in a drop of saline so that they could be counted and examined to determine that all were in approximately the same stage of development. They were then flushed from the corner of the cover glass into the abdominal cavity of another previously uninfected cotton rat. The incision through which the worms were introduced was closed in 2 layers with cotton sutures. All lesions healed satisfactorily with a minimum of scar tissue and without evidence of bacterial infection. The period from infection to autopsy of the donors will be spoken of as the "age" of the worms. Each recipient received similar 7-day-old worms on 3 separate occasions at intervals of approximately 7 days. These recipient animals will be called rats "immunized by worms".

Infective larvae were administered surgically to a second series of cotton rats in exactly the same manner, on a parallel time schedule, and in numbers approximately equal to the number of 7-day worms transferred to the first series of animals. The animals of this second series will be called rats "immunized with larvae".

From 33 to 41 days after these 2 series of animals received their third infection, each rat was given a challenging or "test" infection by introducing a counted number of infective larvae subcutaneously. At the same time previously uninfected cotton rats of a third series were given infective larvae subcutaneously in numbers approximately equal to the number given in the test infections. Animals in all 3 series were killed either 23 or 24 days after the test infection as will be indicated below. The adult worms from the immunizing infections were

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recovered, counted and measured. The worms of the test infections were easily disinguished from those of the immunizing infections and were fixed, mounted and measured by methods described by Westbrook and Scott (1955).

The rats and worms used were of two strains. The rats of 1 strain were from our laboratory-bred colony of *Sigmodon hispidus texianus* and the corresponding strain of worms was derived from worms harbored by the rats from which the colony was established. The other rats were from the Tumblebrook Farms colony of *Sigmodon hispidus hispidus* and the corresponding strain of worms was derived from the same subspecies of rats from Sarasota, Florida (Scott *et al*, 1957). Each recipient rat and its corresponding donors were always of the same strain and the corresponding strain of worms was used for immunizing and challenging. Except for 1 case noted below, all comparisons are made between rats of the same strain.

RESULTS

The criteria used to determine the immunizing effect were the mean length of the worms of the challenging infection and the percentage which had at least started the final molt as compared with similar measurements of the worms from the control animals. The data for the various series of cotton rats are shown in Table I.

TABLE I.—*The number of 7-day worms or infective larvae transferred for immunizing, the number of test (challenging) larvae given, and the percentage of each group recovered*

Protocol	Number of Cotton rats	Mean number immunizing Worms (W) Larvae (L)	Mean number immunizing worms recovered	Mean number test larvae given	Mean number test larvae recovered
A 1	2	207 W	40	85	56
A 2	2	192 L	9	75	59
A 3	1	Control	0	50	60
B 1	2	140 W	49	90	35
B 2	3	212 L	10	83	42
B 1	2	Control	0	99	20
B 2	3	Control	0	86	38

It was not possible to keep all factors as strictly parallel as was desired. The large number of donors needed and the necessarily precise timing of infections created the insurmountable problem of having available at critical times enough mites infected with the proper strain of worms. The first 5 animals listed (Protocol A) were killed when the test worms were 24 days of age. The first 2 animals shown as immunized with 7-day worms (A1) were of the eastern strain and the worms were of the corresponding strain. The second 2 animals immunized with larvae (A2) and the worms used were of this same strain, but a different colony of mites had to be used for the test infections. The mites had, however, been fed on the same infected cotton rat. One of the unfortunate but not unusual occurrences when mite colonies yield few infective larvae prevented the use of exactly parallel controls. Consequently 1 control cotton rat of the Texas strain infected with the corresponding strain of worms was used (A3). A forthcoming report on an extensive series of comparisons between these 2 strains will show that they do not exhibit differences of a degree which would invalidate conclusions in this case. Furthermore, data from a large number of similar control animals from previous experiments are available for comparison. All the evidence indicates that the discrepancies noted do not invalidate the conclusions which will be drawn from these data in the discussion below.

The remaining 10 cotton rats listed in the table (Protocol B) were all killed 23 days after the test infection. The first pair of this group, *i.e.*, those immunized with 7-day worms, were exactly parallel in every respect to the first 2 control animals listed (B1). The 3 animals immunized with larvae were exactly parallel to

the last 3 controls listed (B2). Comparisons of the detailed figures on which this part of the table is based show that these 10 animals can be treated as a unit.

In Table II the measurements and determinations have been pooled by protocols

TABLE II.—*Statistical comparison of pooled values of mean length and percentage at least starting to molt, for worms from animals immunized by 7-day worms, from animals immunized by infective larvae, and from unimmunized animals.* (Length in tenths of millimeters, number measured in parenthesis)

	1 Immunized by worms	2 Immunized by larvae	3 Unimmunized controls	1 – 2 Worms minus larvae	3 – 1 Control minus worms	3 – 2 Control minus larvae
Protocol A:						
Mean length, males	54 (44)	49 (47)	73 (13)	5*	19	24**
Mean length, females	77 (27)	65 (41)	100 (17)	12**	23**	35**
% molting, males	33 (48)	29 (47)	92 (13)	4	59**	63**
% molting, females	11 (28)	0 (40)	24 (17)	11	13	24**
Protocol B:						
Mean length, males	64 (31)	50 (59)	58 (48)	14**	– 6	8**
Mean length, females	95 (30)	56 (47)	78 (72)	39**	– 17	22**
% molting, males	84 (31)	8 (59)	44 (48)	76**	– 40	36**
% molting, females	27 (30)	0 (47)	15 (72)	27**	– 12	15**

* Difference statistically significant at the 5% level.
** Difference statistically significant at the 1% level.

for animals treated alike. Statistical evaluation of the differences between means has made use of the one-tailed "t" test (Dixon and Massey, 1951). For similar evaluation of the differences between percentages Fisher's exact method has been used except that, where the numbers were sufficiently large, one-tailed probabilities from the Chi square test with Yates' correction was used (Fisher, 1932).

The fact that all differences in the last column of Table II are significant at the 1% level indicates that by both criteria in both protocols infective larvae introduced intraperitoneally produced a high degree of immunity. The effect of immunization with transferred 7-day worms, however, was different in the 2 protocols as shown in the next to the last column of the table. In Protocol A the differences were positive and significant at the 1% level except for the percentage of females which had started to molt. In other words a significant amount of immunity was produced by the worms transferred, but it was not as high a degree of immunity as that produced by infective larvae, as can be seen from the figures in the third column from the right. In Protocol B, on the other hand no immunity was produced by the transferred worms. It seems surprising that all of the values in the second column from the right are negative. From a statistical point of view these negative values should be considered as simply showing no difference, since a one-sided hypothesis was adopted, namely that previous infection might retard the growth. In view of the knowledge from our previous experiments that there are multiple factors causing a high degree of variability, it seems reasonable to suppose that the chance concurrence of several of these factors might produce negative numbers of the magnitude observed.

The principal question is why we obtained an expression of immunity in one protocol and not in the other. The most obvious conclusion is based on the fact that in Protocol A an average of 200 worms per animal was transferred, while in Protocol B the average was only 140 worms. It is apparent that we were working near the threshold of immunity, but in view of the variability it would not be wise to indicate an exact threshold value without further experiments.

It appears safe to draw the conclusion that the transfer of a sufficient number of

7-day worms will produce retarded growth of worms of a subsequent infection, but that this effect is not nearly as great as that produced by an equal number of infective larvae.

DISCUSSION

In a previous series of experiments (Scott *et al*, 1956) we arrived at conclusions concerning immunity produced by fourth-stage larvae and young adult worms similar to those reached here concerning advanced third-stage larvae. The infective larvae appear to be much more effective than any of the more advanced stages in producing immunity measured by retarded growth of superimposed infections. One hypothesis regarding the mechanism of this type of immunity is that it is based on an antigen-antibody reaction. If this hypothesis is true it would appear that the antigens produced by the later stages differ from those produced by the infective larvae either qualitatively or quantitatively.

An alternative possibility is that the antigens do not differ so much, but that they are more effective when produced by worms migrating through tissues. In this connection it should be noted that 43% of the 7-day worms transferred were recovered as adults, but only 9% of the infective larvae introduced into the abdominal cavity were recovered. This low recovery of the latter group was a great surprise since about 50% of infective larvae introduced subcutaneously are usually recovered. In spite of the low survival rate, however, a high degree of immunity was produced. Similar results have since been obtained in another series of experiments done for a different purpose. These results raise the question as to whether the infective larvae migrated more extensively than the 7-day worms and thus made more intimate contact with the tissues.

The answer to this question seems to depend on the unknown fate of the worms which were not recovered. In Protocol A, for example, of the 415 7-day worms transferred, 164 were still in the abdomen, 2 were in the thorax, leaving 249 unaccounted for. It is probable that some of the missing worms failed to recover from the effects of handling in transfer. Dead worms of this size would seldom be found at autopsy. On the other hand when 7-day-old worms are introduced subcutaneously, about 2% reach the pleural cavity and the rest cannot be found. Evidently their ability to migrate at this stage is limited. In comparison 384 infective larvae were introduced intraperitoneally in the rats of this protocol; of the 34 recovered, 12 remained in the abdomen and 22 were in the pleural cavity. The missing worms of this group may have died in the abdomen, or they may have started to migrate and since they were following an abnormal route, they may have become lost. The most that can be said is that it seems probable that there was more migration on the part of the infective larvae. It is therefore not possible to conclude whether the observed differences in immunity were due to a difference in antigens in the 2 stages of development or to a differential effect of migration. Attempts will be made to design further experiments to elucidate this point.

SUMMARY

When larvae of *Litomosoides carinii* which have developed 7 days in a cotton rat are introduced surgically into the abdominal cavity of previously unexposed cotton rats on 3 successive occasions, the worms of a subsequent superimposed infection are somewhat retarded in growth and development as compared with the

growth and development of worms of parallel primary infections. This retardation is apparently not as great as that produced by infective larvae similarly introduced. A greater number of the older larvae appears to be necessary for a threshold effect. Whether these results can be explained by differences in type or amount of antigen or by differences in the propensity of the 2 stages to migrate through tissues is an open question.

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RESEARCH NOTE

COELACANTHS AND MONOGENEIDS

In the spring of 1956 I received a pair of formalin-preserved gill arches from specimen C^6 of the coelacanth *Latimera chalumnae* from Dr. Jacques Millot, Director of the Institute for Scientific Research of Madagascar. This specimen had been captured early in February, 1956, near the port of Itsandra, not far from Moroni, the capital of Grande Comore Island.

The branchial material was carefully examined under reflected light using the stereoscopic microscope. This initial search yielded several larval specimens of the gnathiid isopod, *Praniza milloti* Monod, 1954 which was reported from the same specimen by Monod (1954), but no monogenetic trematodes. A more careful examination of each demibranch, lamella by lamella, was conducted. Finally the gills were scraped and the centrifuged detritus was inspected using the stereoscope and transmitted light. Several more isopod larvae were recovered, but no monogeneids.

These negative results were disappointing, but not unique, because Dr. Millot himself stated that gills of several other coelacanths (C^3, C^4, and C^5), also captured in good condition, were examined very carefully for parasites without success (Monod, 1954, Mem. Inst. Scient. Madagascar, Ser. A, **9**: 91–94). Evidently these specimens did not even bear the isopods which were so numerous on C^6. Because of the great zoological importance of the fish, it seems unlikely that these workers would have overlooked any minute gyrodactylids or other parasites possibly present on the specimens. Thus far, then, 3 out of 4 coelacanths have yielded no gill parasites. The single parasitized specimen bore larval isopods only.

Negative results probably can never be taken as definitive evidence that a potential host-species is not parasitized; however, as the number of known uninfected individuals increases, the likelihood that the species is not regularly parasitized by a particular animal also increases. Should this situation be detected in the coelacanth we may have some circumstantial evidence that monogeneids are relatively recent acquisitions of fishes. Other "relict" fishes should also be studied for evidence of sclerotized hard parts, *e.g.* anchors and hooks, clamp and sucker sclerites, and cirral elements, of monogeneids which may yield further information concerning their paleontological history. Other techniques, such as microradiography of fossilized fishes, also may be useful. Similar treatment might be employed to detect fossilized hooked cestodes and acanthocephalans.

In view of the great scientific value that host-parasite data would possess, it is hoped that studies of fresh coelacanths and other relict species will yield more promising results. (Contribution No. 78 from the Virginia Fisheries Laboratory.)—William J. Hargis, Jr., *Virginia Fisheries Laboratory, Gloucester Point, Virginia.*

THE OCCURRENCE OF EGGS IN MALE PHYSALOPTERIDS AND OBSERVATIONS ON THE MORPHOLOGY OF *ABBREVIATA SONSINOI* (V. LINSTOW, 1895) SHUL'TS, 1927

F. G. Tromba,* M. B. Chitwood,* and C. E. Yunker**

The finding of eggs in functional male nematodes is a rare occurrence; we know of only one previous observation. McIntosh (personal communication) has seen eggs in the intestine of small strongyles collected from the cecum of the horse. Our material, all physalopterids belonging to 3 genera, was collected from several hosts in widely separated locations. Two collections, *Physaloptera* sp. from *Citellus leucurus* in Utah and *Skrjabinoptera phrynosoma* from *Leiocephalus carinatus punctatus* in the Bahamas, were sent to one of us (M.B.C.) for identification. A third collection, *Abbreviata sonsinoi*, was made by one of us (C.E.Y.) from *Agama mutabilis* in Egypt.

The systematic position of *A. sonsinoi* has been reviewed by several workers. Described as *Physaloptera sonsinoi* by von Linstow (1895), it was later placed in *Abbreviata* by Shul'ts (1927) after Travassos (1920) had erected the genus. Morgan (1945) reviewed the genus and noted that *A. sonsonoi* [sic] lacked a complete description and could not be classified. Chabaud (1956) commented that the cuticular ornamentation of the male was not typical of other *Abbreviata* from *Agama*. Since a description of the teeth was lacking, Chabaud provisionally placed *A. sonsinoi* in *Skrjabinoptera*.

Having abundant material from the type host, we have examined the heads of several specimens. The denticulation observed in *en face* view (Fig. 6) is that of *Abbreviata*. Since our specimens agree with von Linstow's description, we accept *Abbreviata sonsinoi* (v. Linstow, 1895) Shul'ts 1927.

In the collection of *A. sonsinoi* (U.S.N.M. Helm. Coll. No. 55610) 4 males were found with eggs. In 2 of the specimens eggs were in the intestine (Figs. 1 and 2), while in the others the eggs were in the rectal glands (Fig. 3). In the collection of *Physaloptera* sp. (U.S.N.M. Helm. Coll. No. 55611) eggs were found in the vas deferens of a single male (Fig. 4). Some difficulty was experienced in clearing this specimen; therefore, it has been partially dissected. In the specimens of *S. phrynosoma* (U.S.N.M. Helm. Coll. No. 55932) eggs were found in the rectal glands and intestine of 1, in the intestine of 2 others, and in the cloaca, intestine, and ejaculatory duct of another (Fig. 5). In all cases the eggs were embryonated.

Subsequently we have examined other male physalopterids from birds, rodents, carnivores, and lizards but have not found any other specimens with eggs.

DISCUSSION

The presence of eggs in a nematode having male characteristics may be considered to be either functional or accidental. Functional females with secondary

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male sexual characteristics (spicules and genital papillae) are known. These intersexes were first described by Meissner (1853) and subsequently by Steiner (1923), Christie (1929), and others. Hermaphroditism or syngony, another association of eggs with male characteristics, is not uncommon among free-living nematodes, and cases have been described by Maupas (1900), Potts (1910), and Honda (1925). However, in both intersexual and syngonic nematodes the organism either is essentially female in character or has the external appearance of a female. In the case of the physalopterids described in this paper, female organs were not present and the external morphology was that of the male. Therefore the presence of eggs must be accidental.

In order for eggs to be present accidentally, they must have been taken in through one of the large orifices of the body. In the case of the male nematode this would be the oral or anal opening. McIntosh (personal communication) has noted that the small strongyles of horses are frequently found to have eggs in the intestine. Indeed, he has observed that the males often have bits of the hosts' ingesta and cecal protozoa as well as nematode eggs in all regions of their gut. In this case it seems reasonable to accept the circumstantial evidence that the eggs were ingested.

In our specimens certain facts led us to postulate that the eggs were not ingested, but drawn or forced into the male during copulation. The location of the eggs in the intestine suggests this view, since none were ever found anterior to the posterior third of the gut. The presence of eggs in the rectal glands and in the male reproductive system could result only from positive pressure forcing the eggs into these locations. It seems unlikely that the intestinal musculature could exert such a force and in any event the pressure would be dissipated through the anal opening.

We envisage a series of events as follows: During copulation a close union is effected by the insertion of the long spicule into the vagina and the clasping of the female by the male tail and alae. Sperm are discharged by the contractions of the ejaculatory duct, which is quite muscular particularly in the genera *Abbreviata* and *Skrjabinoptera*. The forcible introduction of sperm would create a positive pressure in the vagina that would not be dissipated through the vulva because of the close apposition in copula and would not be dissipated through the uteri because of the presence of eggs. The anal opening is exceptionally large in physalopterids, and, consequently, when the ejaculatory duct relaxes and a pressure gradient is established, eggs could easily be forced into the cloaca of the male and thence into the rectal glands, intestine, ejaculatory duct, and vas deferens by the positive pressure in the vagina. It is probable that some eggs are forced further up into the intestine and also the vas deferens by the contraction of the spicular muscles when the spicules are retracted. Eggs have been found most commonly in the intestine and less frequently in the rectal glands, the cloaca, ejaculatory duct, and the vas deferens. The explanation of this is that an impediment exists at 2 points: (1) the valve at the junction of the vas deferens proper and the ejaculatory duct; and (2) the narrow duct connecting the rectal glands with the cloaca. It is possible that we have not found eggs more frequently in the ejaculatory duct because their presence may act as an irritant and they are quickly expelled. Since eggs in the cloaca would be subject to movement either up the intestine or out the anal opening, we would expect their presence in the cloaca to be transitory.

Although the evidence is circumstantial, we believe that the foregoing explana-

tion best fits the observed conditions. It may be argued that the mechanism of copulation is essentially similar among nematodes and that if the presence of eggs is a result of copulation, males with eggs should be found frequently. Furthermore, if the eggs are present because of ingestion, it also should be a fairly common occurrence. The reason that it may be a unique or at least rare phenomenon is that a combination of conditions, of which 3 are in themselves uncommon, must occur. The first is that the male must copulate with a gravid female. Insofar as we know, the copulatory behavior of nematodes has not been studied sufficiently to determine definitively whether single or multiple matings are the rule. One of us (M.B.C.) has observed that multiple matings take place in *Rhabditis strongyloides*, but we know of no observations on other genera. In some cases multiple matings are unlikely or impossible because of the short life span of the male, of the separate locations of the sexes in the host, or of the actual blocking of the vulva by a plug. On the basis of analogy with other short-lived invertebrates, it would appear that single mating is the rule and multiple matings are the exception. The second condition is that the male possesses an ejaculatory duct of sufficient muscularity to produce the requisite pressure. The physalopterids are exceptional in this respect since according to Chitwood and Chitwood (1950) the ejaculatory duct in the Phasmidia is in general sparsely provided with muscles. Furthermore, the aforementioned presence of a large anal opening is certainly favorable for the passage of eggs. Finally the absence of a gubernaculum or any accessory structures would facilitate the ingress of eggs.

We therefore postulate that since the finding of eggs in male nematodes is rare, some special conditions must exist to cause this phenomenon. In our specimens the location of the eggs precludes the possibility of ingestion. However, in physalopterids certain anatomical details favor the introduction of eggs into the male during copulation.

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EXPLANATION OF PLATE

FIGURE 1. *Abbreviata sonsinoi* (×40). Eggs in the intestine.
FIGURE 2. *Abbreviata sonsinoi* (×200). Eggs in the intestine.
FIGURE 3. *Abbreviata sonsinoi* (×200). Eggs in rectal glands.
FIGURE 4. *Physaloptera* sp. (×40). Eggs in vas deferens.
FIGURE 5. *Skrjabinoptera phrynosoma* (×200). Eggs in cloaca, intestine, and ejaculatory duct.
FIGURE 6. *Abbreviata sonsinoi* (×500). Head, *en face* view.

RESEARCH NOTE

THE MIRACIDIUM OF *WARDIUS ZIBETHICUS* (TREMATODA: PARAMPHISTOMATIDAE)

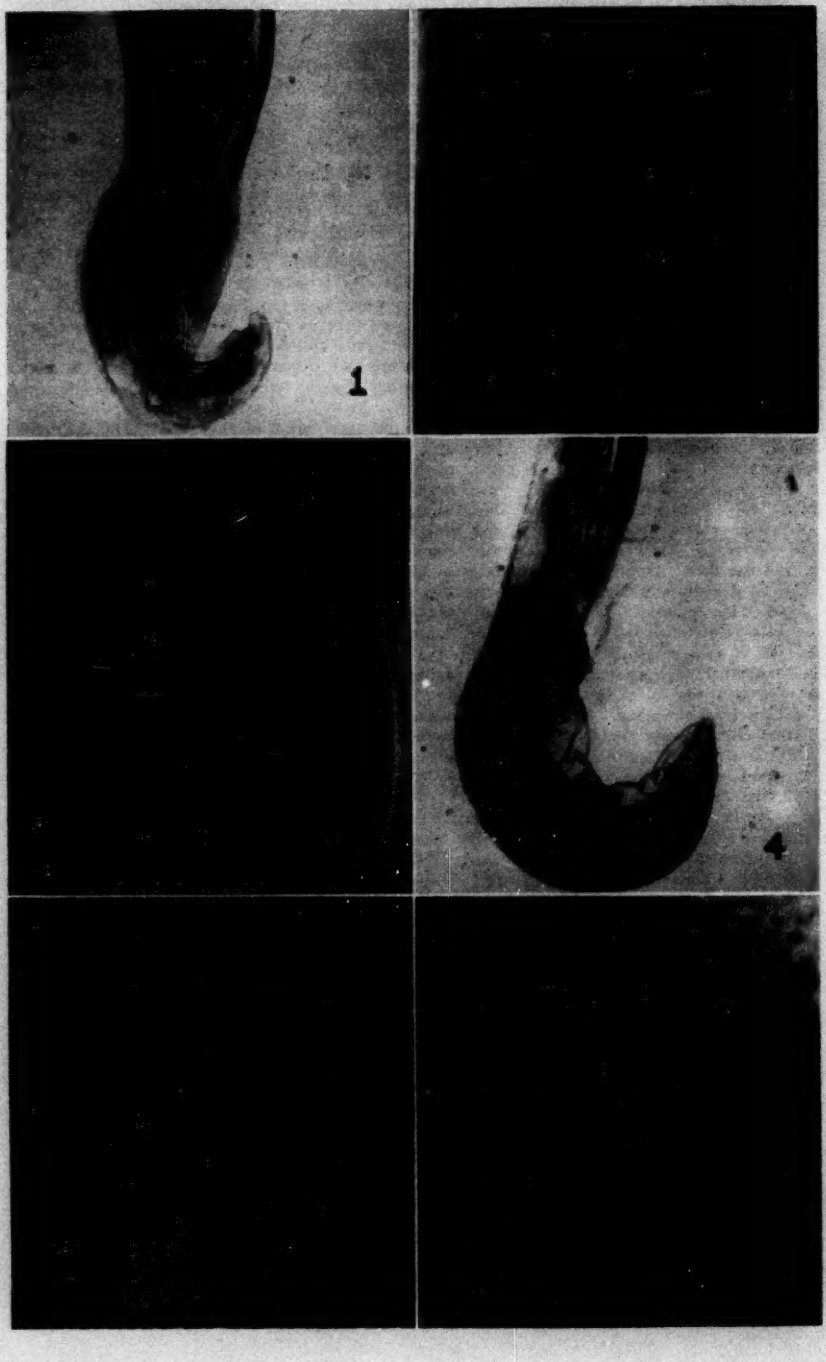
One hundred and twenty five muskrats were trapped from 2 marsh areas 12 miles west of Ann Arbor, Michigan. Sixty-two were infected with 1 to 16 large amphistome trematodes *Wardius zibethicus*, in the cecum. Observations were made on developing miracidia in eggs dissected from the uteri of the worms. Eggs were kept in dishes containing 0.5% potassium dichromate to reduce the destructive action of bacteria. One hundred eggs per dish were kept at laboratory temperatures until hatching. During the winter months the incubation period was from 45 to 50 days. Some miracidia required slightly longer for complete development. The egg shell is smooth except for a small posterior knob. An operculum with inner serrations is present. These unite with serrations along the outer edge of the shell. The operculum seldom becomes disengaged after hatching. In the single-cell stage, the nucleus is in the anterior third of the ovum. Eggs are usually deposited in the 2- to 4-cell stage. Between 60 and 70 vitelline cells fill the shell. Maintained at room temperatures, the embryo is usually in the 16-cell stage of development 6 days after removal from the worm. These cells are not all the same size. At the end of the 2nd week the embryo is oval and irregular in outline. By the end of the 4th week of development, the embryo is elongate and occupies half the space within the shell. One-fourth of the original number of vitelline cells are still intact. At this stage of development, the contents of the eggs show a distinct partitioning through the middle plane. This is outside the embryo and probably is due to a coalescence of the vitelline cells. Several bright red or green refractive concretions, apparently excretory products, always appear at various places between the embryo and the shell. The embryo becomes slightly coiled at this stage and exhibits periodic writhing and shifting movements. After 4 weeks of development, the apical gland is recognizable. It has definite movement and varies in size with the degree of extension. During the 5th week of development the apical gland enlarges and dermal cilia become visible. Those surrounding the sensory papillae are longer than others. There is no ciliary movement while the embryo is in the shell. Throughout the 6th week, the increased length of the miracidium causes it to assume a J-shape. Germinal masses and flame bulb cilia appear distinctly.

Developing miracidia maintained under varying laboratory temperatures throughout the winter months, hatch sometime during the latter part of the 7th week. Hatching is preceded by vigorous movements within the shell. There appeared to be no means of escape other than force. Once the operculum is open, escape of the miracidium is then a matter of minutes. Although swarm hatchings occurred chiefly during the late evening hours, application of heat or light seemed to have little effect upon the development or escape.

Mature miracidia of *Wardius zibethicus* are similar to those described for other amphistomes such as *Cotylophoron cotylophorum*, *Olveria indica* and *Zygocotyle lunatum*. Movement ceases after about 15 hours. Fifty specimens fixed in warm formalin had a mean length of 267 microns and a mean maximum width of 77 microns. From anterior to posterior the dermal cells number 6:8:4:2. The large coarsely granular apical gland fills nearly 1/4th of the body. A small duct leads from the anterior portion of the gland to the tip of the terabratorium. A number of small openings are present through which apical secretions exude. Efforts were made to see other anterior glands. None was observed. A large mass of nerve tissue occupies a dorsal position near the anterior end of the miracidium. In living forms small nerve fibers could be seen which extended to lateral sensory papillae. The excretory system is similar to those of other described amphistome miracidia. Two large flame bulbs are present with ducts leading anteriorly then posteriorly and emptying through 2 small pores situated posteriorly. The posterior 2/3rds of the miracidium is filled with germinal masses. The largest of these consists of a cluster of closely packed cells, probably an embryonic mother redia. This lies just back and nearly touching the apical gland. Other smaller germinal masses are scattered posteriorly.

Penetration experiments were carried out involving several species of laboratory-raised snails. None of these was successful.—R. JAY SMITH, *University of Detroit.*

PLATE I



EFFECT OF FOUR EMBRYONATION MEDIA ON THE EMBRYONATION AND INFECTIVITY TO CHICKENS OF *HISTOMONAS*-BEARING EGGS OF *HETERAKIS*

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Since the discovery by Graybill and Smith (1920) that the cecal worm, *Heterakis*, plays a role in the transmission of blackhead (histomoniasis) in turkeys, many investigators have studied the development of the worm, or have fed embryonated eggs of the cecal worm as a means of inducing blackhead in chickens and turkeys.

A review of the literature indicates that the media most commonly used for the embryonation of *Heterakis* eggs have been (1) physiological saline, (2) weak solutions of nitric acid, (3) weak solutions of formalin, and (4) a 2.0% solution of potassium dichromate. Almost no data are given as to the proportion of eggs which embryonated in each medium, and in most instances the number of worms recovered at necropsy in proportion to the number of embryonated eggs given must be calculated by the reader. The percentage of recovery has usually been low.

The present study was designed to determine the relative merit of each of 4 culture media, namely, physiological saline, 1.5% nitric acid, 1.0% formalin, and 2.0% potassium dichromate, for the embryonation of *Heterakis* eggs, and what effect, if any, each medium might have on the subsequent development of the worms in the host and on the ability of those worms to transmit histomoniasis.

MATERIALS AND METHODS

Seven hundred and thirty female worms 30 to 44 days old and of comparable origin were divided arbitrarily into 4 approximately equal groups. Those in each such group were placed in a shallow culture dish containing one of the aforementioned media, and maintained at approximately 72° F until embryonation appeared to be complete (18–19 days).

The worms comprising each lot were then washed in several changes of tap water and then in several changes of physiological saline. This washing removed most of the embryonation medium and practically all the debris. The eggs were liberated by splitting open the female worms and teasing apart the uteri. Coarse particles were washed free of adhering eggs and removed with forceps or a pipette, and the eggs were then washed in several changes of physiological saline to remove the debris and any of the embryonation fluid that might have remained inside the worms. The percentage of embryonation was then determined by counting both embryonated and unembryonated eggs in several 0.1-ml samples of the agitated suspension.

Immediately before the eggs were administered to the test birds, each of the 4 suspensions of eggs was calibrated by counting the number of embryonated eggs in each of 4 samples of 0.2 ml, averaging the counts, and adjusting the volume of the suspension so that 1 ml contained approximately a calculated 155 embryonated eggs.

In total, 240 purebred New Hampshire Red chickens, 5 weeks old, which had been raised under parasite-free conditions were used as test birds. They were divided into 4 groups of 60 birds each. Each bird then received, by means of a pipette, 1 ml of the suspension of eggs embryonated in the medium being tested with the 60 birds in that group.

After infection, the test birds were held under conditions which precluded the acquisition of extraneous infections of either cecal worms or histomonads. Six birds from each group were autopsied for study on each of the following days: the 10th or 11th, 14th, 17th or 18th, 21st, 28th, 35th, 42nd, 49th, 56th, and 63rd. Insofar as the present study is concerned, the post-mortem examination of each bird consisted of an examination of the ceca and liver for evidence of lesions

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due to *Histomonas*, of smears of the cecal contents for the presence of histomonads, and of the ceca for heterakids. All the cecal worms were collected and counted and 10 of each sex, or as many as were found up to that number in each bird were measured.

RESULTS

The percentage of eggs which embryonated in each of the media was as follows: 27.3 in physiological saline, 25.2 in nitric acid, 31.0 in formalin, and 28.6 in potassium dichromate.

Data on the number of *Heterakis* worms that were recovered at designated periods following the administration of embryonated eggs are shown graphically in Figure 1.

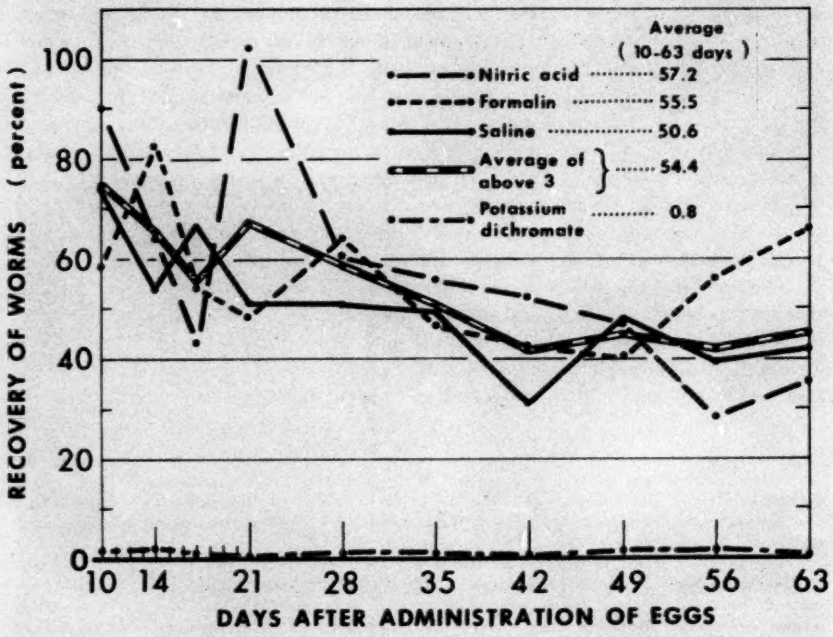

FIGURE 1. Recovery of *Heterakis*, according to medium used for embryonation of eggs, and the time of examination.

Histomonas organisms were frequently found in the cecal contents of chickens on this experiment, but no cases of clinical histomoniasis were observed at any time.

Previous experience has shown that *Histomonas* cannot be detected as readily in cecal smears of birds killed during the first 2 weeks after inoculation with *Heterakis* eggs as it can be later. Consequently, the frequency with which *Histomonas* was found is presented separately for the entire period (from the 10th to the 63rd day) and for the period covering the 17th to the 63rd day of observation.

Histomonas was detected in 83% of the birds which had received *Heterakis* eggs embryonated in physiological saline, and in 91.5% of the 47 birds which had been killed and examined from the 17th to the 63rd day after infection. The corresponding figures for birds infected with eggs embryonated in 1.5% nitric acid solution were 75% and 83.3%, respectively, whereas those for birds receiving eggs

that were embryonated in 1.0% formalin solution were 86.4% and 93.6%, respectively. The lowest incidence of histomonads occurred in the birds fed eggs embryonated in potassium dichromate solution. Only 6.8% of the 60 birds autopsied during the entire observation period and only 8.5% of those examined between the 17th and 63rd days after infection contained *Histomonas*.

Both male and female worms from eggs embryonated in all 4 media were of normal size for their age at all periods of observation.

DISCUSSION

On the basis of the findings reported herein, physiological saline, 1.5% nitric acid, and 1.0% formalin are satisfactory media in which to embryonate eggs of *Heterakis*. When physiological saline is used, care must be taken to keep molds and bacteria from becoming excessively numerous, or many eggs may be lost. The larvae of some arthropods consume large numbers of eggs in saline if they gain access to the cultures.

The percentage of eggs which embryonated in each of the 4 media is considered satisfactory for worms of the age used. Worms of 30 to 44 days of age contain large numbers of eggs which have apparently not been fertilized, but which have developed sufficiently to persist and be counted. The percentages of eggs that embryonated in each of the 4 media are regarded as comparable.

The proportion of worms recovered with respect to number of embryonated eggs administered (a calculated 155 to each bird) fluctuated somewhat at different periods of observation, because only 6 birds in each lot were killed on any one day. But as Figure 1 shows, the averages for the entire period are quite similar for all the media except the 2.0% solution of potassium dichromate, for which worm recovery never exceeded 1.5%, and which averaged only 0.85% for all 10 periods of observation. This is in marked contrast to the overall recovery of 50.6% from eggs embryonated in saline, 55.5% for those embryonated in 1.0% formalin, and 57.2% from eggs embryonated in 1.5% nitric acid. For the latter 3 culture fluids the total worm recovery averaged 54.4%, or 64 times that for eggs embryonated in 2.0% potassium dichromate.

The larvae within the eggs embryonated in 2.0% potassium dichromate were as active on stimulation by warming as were those of eggs embryonated in any of the other media. Moreover, they remained alive and could be stimulated to show normal activity as long as 4 months after embryonation, at which time testing was discontinued. However, the shells of the eggs were very tough, as evidenced by their failure to rupture when pressure was applied to a coverglass overlying them. Eggs embryonated in the other fluids split open readily when subjected to this treatment, and liberated active larvae. It is believed that this toughness of the shells of eggs embryonated in 2.0% potassium dichromate prevented the hatching of many eggs in the birds.

Histomonas was detected in 8.5% of the birds which had received eggs embryonated in 2.0% potassium dichromate 17 to 63 days prior to being killed for study. This is less than 1/10th the incidence recorded for birds of the other 3 lots for the same period, but still stands in marked contrast to the 1 to 64 ratio for worms recovered from these lots, grouped the same way.

Histomoniasis was present in birds in the potassium dichromate lot about 6 times as frequently in proportion to worms recovered as was the case in the other 3 lots.

Two circumstances may have operated to this end. *Histomonas* may have been present in a somewhat larger number of eggs than was required to produce the infections recorded; and more of the eggs embryonated in the dichromate solution may have hatched and developed far enough to liberate *Histomonas* than were able to continue development long enough to be recovered at the times the birds were killed. These larvae may have depleted their energy reserves to such an extent in escaping from the tough shells that only limited development was possible.

SUMMARY

1. A study was made of the effects of 4 embryonation media, namely physiological saline, 1.5% nitric acid, 1.0% formalin, and 2.0% potassium dichromate, on the embryonation of *Heterakis* eggs, the subsequent development of the worms in chickens fed eggs so cultured, and the ability of the eggs or worms to transmit *Histomonas*. Sixty birds were used to test eggs embryonated in each of the 4 media.

2. A 2.0% solution of potassium dichromate permitted satisfactory embryonation (28.6%) in 19 days at room temperature. However, the number of worms recovered from chickens fed these eggs averaged less than 1% of the number of embryonated eggs administered, and infections with *Histomonas* were detected in only 8.5% of 47 birds killed and examined 17 to 63 days after each had received 155 embryonated *Heterakis* eggs.

3. Physiological saline, 1.5% nitric acid, and 1.0% formalin permitted embryonation of 27.3%, 25.2%, and 31.0% of the eggs, respectively. Worm recovery from eggs embryonated in these media was comparable, and for the 3 groups combined, it averaged 74.3% in chickens killed at 10 and 11 days, 45.1% in those killed at 63 days, and 54.4% for those killed at all periods of observation. *Histomonas* infections were detected with comparable frequency following administration of *Heterakis* eggs embryonated in any one of the 3 media. Of 142 chickens examined 17 to 63 days after each had received orally 155 embryonated *Heterakis* eggs, 89.4% were found to harbor *Histomonas*.

REFERENCE

GRAYBILL, H. W. AND SMITH, THEOBALD 1920 Production of fatal blackhead in turkeys by feeding embryonated eggs of *Heterakis papillosa*. J. Exp. Med. 31: 647-655.

EFFECTS OF THE SCREW-WORM ON GUINEA PIGS

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Callitroga hominivorax, the screw-worm, is well recognized as a cause of weight losses in cattle and other domestic animals. Borgstrom (1938) reported weakness, emaciation, and loss of appetite in guinea pigs infested with this parasite; these symptoms were less severe in reinfestations. Laake and Smith (1939) confirmed these findings and reported that abortion usually resulted in guinea pigs with heavy infestations (2 larvae per 100 g body weight). Borgstrom stated that by the 5th day of infestation the average leucocyte count had dropped from a normal of from 10,000–13,000 to 5,800–7,000, averaging 6,500. After this time the leucocyte count rapidly rose and became normal on the eighth day. Micks and Calma (1952), on the other hand, reported an elevation in white blood cell counts in human cases of nasal infestation by the screw-worm. In these cases, temperature peaks of 103° to 104° F are also common.

Borgstrom described a gram-negative bacillus, *Proteus chandleri*, which she found to be invariably present, and nearly always in pure culture, in lesions infested with *C. hominivorax*. Although the function of the organism is not known, the bacteria were reported to have no relation to the production of the immunity developed to effects of the screw-worm.

It can be seen that there is little consistent or detailed knowledge concerning the symptoms evoked by these myiasis-producing flies, and even less concerning the mechanisms by which they are produced. The purpose of this work is twofold: first, to elucidate the clinical picture manifested by screw-worm infestations; and second, to provide a basis for investigations into how the symptoms are produced, with special reference to the production of toxic substances, and the possible development of antitoxic reactions that are protective to the host when reinfestations occur. The present paper deals with the first of these matters.

My sincere appreciation is expressed to Dr. Asa C. Chandler under whose direction this investigation was carried out. Thanks are also given to Mrs. Eveyln Hake for her valuable technical assistance. This study was supported by a grant (No. E-876) from the National Institutes of Health.

MATERIALS AND METHODS

The laboratory stock of *C. hominivorax* was started from pupae kindly sent by Dr. R. C. Bushland and Mr. C. L. Smith at the U.S.D.A. Entomology Research Branch at Kerrville, Texas. The techniques employed in maintaining the stock culture were for the most part the same as those practiced at Kerrville (Bushland and Hopkins, 1953). The medium used for rearing the larvae was a modification of that formulated by Melvin and Bushland (1940). Its constituents were finely ground lean horse meat, 500 g; citrated human blood (out-dated blood-bank stock), 250 ml; formalin, 3 ml; and water, 500 ml. Larvae were incubated in this medium at 37° C. For experiments, eggs were obtained by inducing females to oviposit in a vial containing a piece of fresh horse meat. The eggs were removed from the walls of the vial and placed in a petri dish on filter paper moistened with saline. The larvae hatched after about ten hours in a 37° C incubator. Larvae so obtained were used to infest the animals.

The guinea pigs used throughout this study were obtained locally from a single source.

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Most were females; exceptions are noted in the groups below. The 41 animals used in this study ranged in weight from 375 to 589 grams with an average weight of 483 grams. These animals were fed Purina rabbit-chow checkers supplemented daily with fresh lettuce.

To investigate the detailed effects of screw-worms on the hosts, attempts were made to produce parasite burdens comparable to those reported by previous workers to cause serious symptoms without resulting in death in most of the animals, i.e., heavy sublethal infestations. Borgstrom (1938) infested guinea pigs of about 450 g with 8 larvae. She found that nearly all animals survived this number, but infestations of 10 or more larvae were usually lethal. Laake and Smith (1939) found that 2 larvae per 100 g body weight was with few exceptions the maximum sublethal dose in their work with guinea pigs. Nearly all animals died from infestations of 3 larvae per 100 g. In the present study, attempts were made to obtain infestations as near to 2 larvae per 100 g as possible. The number of larvae which succeeded in establishing themselves in a wound was found to be variable, in the work by Borgstrom and by Laake and Smith, and in this investigation. In the 32 infested animals involved in the present study, an average of 60% of the larvae became established and grew to maturity in the lesions, the range being from 0 to 39 larvae.

Prior to infestation, animals were anesthetized with ether, and an area on the left shoulder was shaved. The wounds were made by cutting out a circular piece of skin 1 cm in diameter just behind the scapula. The underlying connective tissue and muscle were also cut in several places. The newly hatched first-instar larvae were counted under a dissecting microscope and transferred to the wound with a flamed needle. The wounds were immediately bandaged with a gauze pad moistened with saline.

Clinical data were taken daily at the same time, beginning 3 days prior to wounding, and continuing in most cases for 3 days after the larvae dropped out. These data included the general conditions of the animals, the bacterial flora of the lesions, weight, rectal temperature, erythrocyte counts, and total leucocyte and eosinophile counts. At intervals of 2 days the amount of chow consumed by the guinea pigs was measured.

The experimental animals considered in this study are placed in the following categories. The number and sex of guinea pigs included in the group are shown in parentheses.

Heavy sublethals: Animals surviving an infestation of more than 1.5 larvae per 100 g, 8 to 37 larvae. (8 females, 3 males; 3 additional males were included for the total leucocyte counts).

Light sublethals: Animals surviving an infestation of 1.5 or fewer larvae per 100 g, 4 to 8 larvae. (8 females).

Lethals: Animals not surviving an infestation of 14 to 39 larvae; 3.2 to 9.5 larvae per 100 g. (6 females).

Normal controls: Animals wounded but not infested. (4 females).

Bacteria controls: Animals wounded and infested, but which lost all larvae before the third day; typical gram-negative bacilli (Borgstrom's *Proteus chandleri*) present. (4 females).

Eosinophile group: Heavy sublethal cases (6 males) and wounded but uninfested controls (3 males) upon which only total leucocyte and eosinophile counts were made.

Weight controls for food: Animals not wounded or infested, but fed the same amount of food as the heavy sublethals to check weight loss due to appetite loss. (5 females).

OBSERVATIONS

1. *Course of Larval Development:* Within an hour after being placed into the wound, the first-instar larvae began to aggregate around the edge. During the first day of infestation a slight amount of clear serous exudate was present. By the second day of infestation, the larvae, then in second instar, had begun to form a pocket underneath the edge of the wound, and the amount of exudate increased. There was little increase in the size of the larvae, which by then weighed only 4.9 mg. By the third day nearly all the larvae had undergone a molt to third instar and had increased in size to about 16 mg. The amount of exudate was greater and the pockets in the tissue were deeper. From the third to the sixth day these third-instar larvae rapidly increased in size, tissue destruction reached a maximum, and the exudate became dark and dripped from the lesion. By the sixth day all larvae were of maximum size (average 120 mg). On the seventh day, as most of the larvae began to drop from the lesion, purulence was observed in some of the wounds. All the larvae had left the wound by the end of the eighth day, and in about half of the animals

TABLE I. *Effect of screw-worm infestation on weight, temperature, and erythrocyte count in guinea pigs.*

Animal (Group)	Sex (No. in Group)	Larvae per 100 g	Weight					Temperature					Erythrocytes				
			Ave. Pre.-Inf. Wt.	Maximum Loss		% Gain or Loss Later		Ave. Pre.-Inf. Temp.	Maximum Temperature			Temp. 2 days after peak	Ave. Pre.-Inf. Count	Minimum Count		Last Count	
				%	Day	%	Day		Temp.	Rise	Day			No.	Day	No.	Day
1	F	2.0	495	10.1	6	− 3.4	10	101.7	105.6	3.6	5	102.0	6.07	2.67	8	4.31	11
2	F	2.0	507	2.8	5	+ 2.9	10	102.4	105.5	2.6	5	102.0	6.13	4.76	8	5.99	10
3	F	3.6	568	26.1	9	− 17.4	12	102.2	104.8	2.3	4	100.0	5.42	4.33	11	4.34	12
4	F	1.6	488	5.9	8	− 0.4	12	101.4	103.4	1.4	4	102.0	6.03	3.69	7	5.37	12
5	F	3.1	441	10.2	7	− 5.0	12	101.8	104.4	2.3	4	103.9	6.02	2.74	6	3.86	12
6	F	2.7	476	19.1	7	− 8.4	12	101.7	104.6	2.8	4	102.5	5.89	3.47	9	5.25	12
7	F	2.5	562	15.5	7	− 9.8	12	101.5	103.8	2.1	5	103.0	5.53	3.73	9	4.40	12
8	F	7.7	487	24.6	8	− 15.8	12	102.2	104.6	1.8	3	103.4	6.13	2.38	11	3.43	12
9	M	1.8	490	27.4	12	− 27.4	12	101.6	104.8	2.9	7	103.0	5.48	3.39	12	3.39	12
10	M	1.6	505	28.3	10	− 26.2	12	102.3	104.8	1.4	6	104.6	6.49	4.20	9	5.04	12
11	M	1.9	453	19.6	6	− 3.9	12	102.1	104.1	1.2	5	103.3	6.25	5.35	10	5.85	12
HS	11	1.6–7.7	497	17.2	7	− 12.7	12	101.9	104.6	2.2	5	102.7	5.95	3.70	9	4.55	12
L	6	3.2–9.5	468	26.3	d	—	—	101.7	104.7	2.9	4	—	6.00	3.66	dd	—	—
LS	8	0.7–1.5	458	3.6	1	+ 4.2	10	101.6	104.2	2.2	5	102.7	6.00	4.50	8	5.14	10
NC	4	0	516	0.9	1	+ 3.1	11	102.2	102.8	0.1	4	102.1	6.23	5.59	2–7	6.29	10
BC	4	0	442	0.5	2	+ 7.5	10	102.0	103.1	0.7	9	102.2	5.92	5.07	5	5.91	10
FC	5	0	512	5.7	3	− 3.4	9										

Above the double line are data for individual heavy sublethals. Below the line data are expressed as means (modes for "day") for heavy sublethals (HS), lethals (L), light sublethals (LS), normal controls (NC), bacteria controls (BC), and food controls (FC). "Rise" means degrees above the highest temperature recorded for an individual prior to infesting or wounding. Erythrocytes are in millions of cells per cmm. For lethals, "d" means at death and "dd" means the day before death.

a thick purulent exudate replaced the fluid exudate in the lesion. By the twelfth day (4 to 5 days after the larvae had dropped out) a scab had formed over the wound.

2. *General Aspects:* Control animals remained firm and active throughout the study. Light sublethal cases became more quiet than usual and were less firm after the fourth or fifth day. Heavy sublethal cases showed a great diminution in activity and marked loss in appetite by the fifth day. These animals became very thin and flaccid toward the end of the infestation. Feces of this group were small and very moist. Lethal cases presented an exaggeration of the conditions in the heavy sublethal group. Activity was less, food intake was lower, and all showed irregular breathing after about the fifth day of infestation. Two animals died on the sixth day while the maggots were still within the lesion, two on the seventh day as the larvae were emerging, and two died on the second day after the maggots dropped out.

Daily streak plates, made on nutrient agar, revealed the invariable presence of the gram-negative bacilli (*Proteus chandleri* Borgstrom, 1938) in the exudate of infested lesions. The organism, after the second day, was usually in pure culture. Lesions of normal control animals were either sterile or possessed staphylococci, but *Proteus* was never found. Bacteria controls revealed *Proteus* up to the fourth day, when the wounds became dry.

3. *Weights:* Gains and losses for each animal are expressed as percentages of the mean of the weights of that individual recorded for the 3 days prior to infesting or wounding. These were found to be more comparable than absolute changes, since the animals differed in weight. Mean weights, as well as other pertinent data for each group, are shown in Table I.

The normal controls showed a mean gain in weight throughout the period corresponding to an infestation, and this was true also of the bacteria controls.

Light sublethals showed a slight mean gain in weight throughout the infestation, although individual animals underwent occasional small losses.

In heavy sublethal cases, definite weight losses began to occur by the third or fourth day and most reached minimum values on the seventh to the ninth days. Mean values for this group are shown in Figure 1.

Food controls, fed the same amount of chow consumed by the heavy sublethals, an average of 27 grams per day, underwent slight but consistent losses. The average of the maximum losses in this group was only 5.7%, as compared with 17.2% in the heavy sublethal cases. The weight loss in the heavily infested animals was clearly not due to loss of appetite alone.

The six lethal cases underwent a weight loss from the first day. By the third day weights began to drop precipitously, the losses at the time of death ranging from 21.3% to 30.6%.

4. *Temperatures:* An oral clinical thermometer (Fahrenheit scale), inserted 4 cm into the rectum, was used to determine rectal temperatures. Data for all groups are seen in Table I.

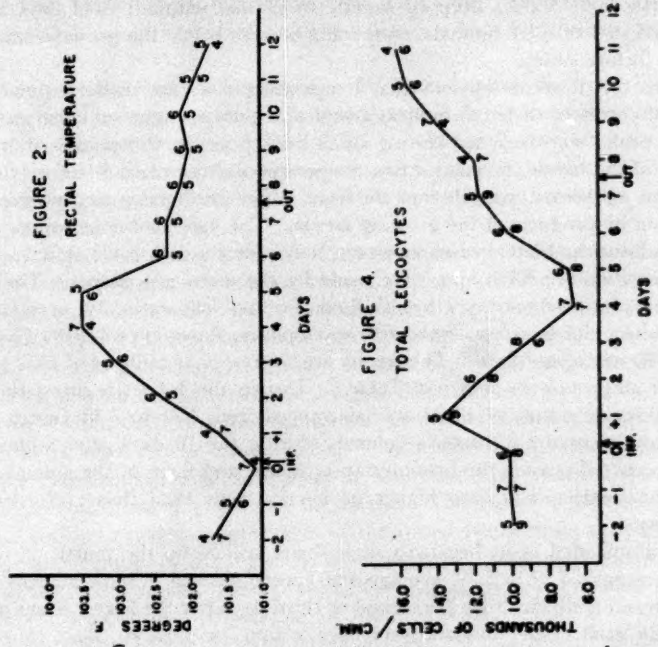
The normal controls and bacteria controls all remained within their pre-infestation range of temperatures throughout the 10 days following wounding.

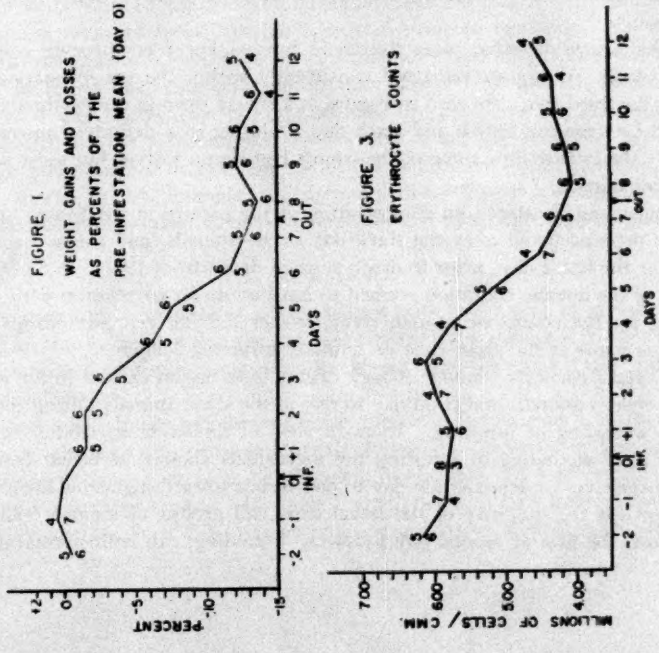
In the light sublethal cases, the temperatures began to rise by the first or second day. Peak temperatures, ranging from 103.2° to 105° F, were reached on the fourth to the sixth day.

The picture of the heavy sublethals is almost exactly the same as that of the light

Course in Heavy Sublethal Infestations

These graphs represent the means of the group for each day. Numbers above and below the line show the number of animals possessing values above and below the mean, respectively. Animals were infested on day zero, immediately after data were taken. Where data were occasionally lacking, values were interpolated whenever possible and incorporated into the mean.





sublethals. An initial rise occurs, with an average peak of 104.6° F reached around the fifth day, followed by a rapid drop back to near or below the normal values about two days after the peak is reached. Figure 2 shows the course in this group.

In the lethal cases, peaks (103.7° to 105.6° F) were reached on the second to the fourth day. Values dropped rapidly to normal within 1 or 2 days after this peak, and in 4 of the 6 animals, temperatures were below the pre-infestation range the day before death.

From this it seems obvious that temperature rises are moderate and are not essentially greater in lethal or heavy sublethal than in light sublethal cases. Although peaks were reached during or a little prior to the period of maximum growth of the larvae, in many cases temperatures dropped back before this phase was over. It seems probable that the fever is not attributable to a progressive intoxication by products of the growing larvae. The drop to below normal, encountered in heavy and lethal cases, however, may indicate such toxic activity.

5. *Blood Picture:* All counts were made by the usual procedures. The diluents used were: for erythrocytes, Hayem's fluid; for total leucocytes, 3% acetic acid with methyl violet; for total leucocytes with eosinophiles, Randolph's (1949) glycol stain.

A. *Erythrocyte Counts:* The counts are expressed as millions of cells per cmm. Data for all groups are shown in Table I. During the 3-day pre-infestation period the erythrocyte counts of all 33 animals ranged from 4.71 to 7.58 (mean 5.95).

In both normal and bacteria controls, during the 10 days after wounding, no values occurred outside the pre-infestation range, and none of the animals showed consistent tendencies toward higher or lower counts than those recorded before wounding.

Light sublethal cases began to show lower counts by the fourth or fifth day. Lowest counts (3.59 to 5.79, average 4.50) were reached in most animals between the sixth and tenth day. By the second or third day after the lowest count occurred, values for each of the animals were back within, or close to, their range before infestation.

In the heavy sublethal cases the trend toward lower erythrocyte counts was more striking. All counts remained consistently within the pre-infestation ranges through the third day. As seen in Figure 3, a steady drop in the erythrocyte count occurred between the fourth and sixth day, resulting in a definitely anemic condition. By the twelfth day, some of the counts had begun to rise, but most were still well below normal.

Lethal cases illustrated an exaggeration of the picture in the heavy sublethals. A sharp drop occurred after the third day in all animals, and the average of the counts for the last 2 days prior to death showed drops from 19.0% to 63.4%. The severity of the anemic condition seemed to have no direct correlation with the time of death, for the counts of animals dying earlier did not represent drops greater than those made at the same time on animals surviving longer.

B. *Total Leucocyte Counts:* A high degree of variation existed in the leucocyte counts between animals and from day to day in the same animals during the 3 days prior to wounding or infesting. Even in view of this wide variability in normal animals, after wounding or infesting the individuals showed sufficient consistency within themselves to demonstrate day to day trends toward higher or lower counts.

Except for the majority of the lethal cases, all groups of animals exhibited a leucocytosis the first or second day following wounding. In both normal and bac-

teria controls the counts had returned to, or slightly above, the pre-infestation level by the second or third day. No further pronounced changes were observed in these groups.

Of the 8 light sublethal cases, 7 showed a drop in the leucocyte count averaging 4,780 (35.4%) below the pre-infestation mean on the fifth and sixth day. By the seventh or eighth day, as the larvae left the wounds, counts returned to normal or above.

The means of the heavy sublethal cases for each day are shown in Figure 4. The average drop in leucocytes from the mean of the 3 days before infesting to the lowest count after infesting was 5,060 (40.2%). One or 2 days after the lowest count was reached in each animal, values returned to normal. In most individuals a marked leucocytosis occurred at the time the larvae began to emerge from the lesion (seventh day).

Of the 6 lethal cases, only 1 exhibited a marked rise in leucocytes on the first day after infesting; all others remained within, or close to, their pre-infestation levels. Lowest counts occurred on the second to the fifth day, representing an average drop of 4,480 (42.6%) below the mean before infesting. Those animals which survived the longest (7 to 10 days) showed a marked terminal leucocytosis which began about the seventh day as the larvae dropped from the lesions.

It seems obvious that the initial leucocytosis can be attributed to the process of wounding alone since it was characteristic of both control and experimental animals. The greatest degree of leucopenia in sublethal cases occurred during the period of most accelerated growth of the larvae. The earlier appearance of the leucopenia and the apparent suppression of the initial leucocytosis in lethal cases (with a greater number of larvae) tends to suggest that the secretions and/or excretions of the larvae, which would presumably be greater from the start in these animals, are at least in part responsible for the leucopenia.

C. *Eosinophiles:* In the 6 heavy sublethal cases on which eosinophile counts were made, a range of from 0 to 3.6% existed prior to infesting. No tendency toward an eosinophilia occurred on the first day after infesting. Between the second and the fourth day eosinophile percentages dropped, all animals reaching zero on at least 1 day during this time, paralleling or just preceding the leucopenia. From the fifth day the percentages rose to within or slightly above the pre-infestation range, and remained there to the end of the experiment (last counts on seventh or eighth day). Control animals ranged from 0 to 6.7% over a comparable period of time and showed no consistent tendencies toward unusually high or low counts at any time.

DISCUSSION

By relating the occurrence of the symptoms with the course of larval development, it can readily be seen that the greatest effects of the parasites fell between the fourth and sixth day of infestation when the larvae were undergoing their maximum rate of development.

In general, the clinical picture was more intense in the animals with the heavier infestations, although this was not strictly followed. Individuals demonstrating severe effects in one aspect (e.g., development of anemia) did not necessarily show parallel effects in other aspects. In Table I it will be seen, for example, that among the heavy sublethals, Nos. 3 and 11 suffered severe losses in weight, but relatively

little loss of erythrocytes, whereas in Nos. 1 and 5, with moderate weight losses, the loss in erythrocytes was exceptionally high.

Since no consistent differences existed between the bacteria controls and the normal controls, it must be concluded that *Proteus chandleri* alone, at least in the amounts normally introduced with an infestation, shows no marked pathological tendencies.

Borgstrom (1938) in her work with screw-worms discarded mechanical damage as the cause of death, since previously infested animals did not die from what would otherwise be a lethal infestation, although there was little difference in tissue destruction. However, in view of the extensive destruction caused by the feeding parasites, this factor cannot be totally dismissed as contributing to the clinical effects.

The picture revealed in the heavy sublethal cases, however, is strongly suggestive of some toxic action on the host, of which at least one effect is a reduction in the blood cells, both erythrocytes and leucocytes. Liberation of decomposition products of the host's tissues might possibly account for the clinical picture, or at least contribute to it, since considerable necrosis is present in heavy infestations. However, the most extreme symptoms occurred during the period of most rapid growth of the larvae, and before tissue destruction and decomposition was at its height. It seems probable that the main toxic agents responsible for the clinical picture and consequent death of the host are to be sought among the metabolic products, secretions and/or excretions, liberated by the larvae. This view is supported by the demonstration of toxic and antigenic properties of the excretory products of other maggots (Blacklock *et al*, 1930; Gwatkin and Fallis, 1938).

In considering the above, it seems likely that the heightened resistance to injurious or lethal effects from reinfestations of screw-worms in the same area as the primary infestation, reported by Borgstrom (1938) and Laake and Smith (1939), may be due to the local neutralization of the toxic products. In this case, the local tissues would become saturated with antibodies before the liberated circulating antibodies could reach an adequate titer, resulting in the neutralization of the toxic substances at their site of production (in the lesion), thereby preventing the toxin from gaining entrance to the general circulation in lethal amounts.

Studies on the nature of the postulated toxic substances and their immunological relationships are in progress.

SUMMARY

Guinea pigs with heavy sublethal infestations of screw-worms (*Callitroga hominivorax*) manifest a marked loss in weight, a rise in temperature, leucopenia and anemia, all of which reach maximum degrees, or experience the greatest change from normal, during the third or fourth to the sixth or seventh days of the infestation, the period during which the third instar larvae undergo their maximum phase of growth. By the time the maggots have left the host (seventh to eighth day) symptoms begin to subside, with the exception of the anemia, which lasts about 2 days longer. In general, animals with lighter infestations exhibit milder symptoms, and in lethal cases the clinical effects were the most severe, but in all criteria of injury investigated there was a remarkable variation in response in different animals, some showing little effect with infestations that were extremely deleterious or lethal

to other animals, and some dying from infestations that other animals tolerated very well.

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RESEARCH NOTE

CROSS TRANSMISSION OF NEMATODES OF DOMESTIC ANIMALS. III. PRELIMINARY OBSERVATIONS ON THE INFECTION OF GOATS AND RABBITS WITH *HYOSTRONGYLUS RUBIDUS*

Douvres and Tromba (1958, Proc. Helminth. Soc. Wash. **25**: in press) reported the experimental infection of a calf with *Hyostrongylus rubidus* of swine. The present communication reports the successful infection of a kid and a rabbit with *H. rubidus*.

An 8-week-old Toggenburg kid was purchased from a local farm and placed in a raised concrete-floored outdoor pen. Salt flotations of the feces were negative for *Hyostrongylus* ova. Nine weeks later 13,000 infective larvae of *H. rubidus* were administered *per os*, and the animal was sacrificed 25 days after infection. At necropsy a heavy natural infection with *Haemonchus* spp. was found along with smaller numbers of *H. rubidus* in the abomasum. The number of *Hyostrongylus* from a portion of the contents and abomasal scrapings was estimated by dilution count to be 2,240 sexually mature adults; the *Haemonchus* were not counted. The infection with *Haemonchus*, which in all likelihood preceded the experimental infection, did not appear to interfere with the establishment of *Hyostrongylus*.

Alicata (1933, J. Parasitol. **20**: 97) experimentally infected guinea pigs with *H. rubidus* and later (1936, Tech. Bull. **489**, U. S. Dept. Agric., Dec. 1935, 96 pp.) used this host to study the parasitic development. As far as the writers are aware, other laboratory animals have not been examined for susceptibility to this nematode.

An 8-month-old New Zealand White rabbit was fed 78,000 infective larvae of *H. rubidus per os* and sacrificed 33 days later. Salt flotation of the feces on the 19th day after infection was negative; however, on the 33rd day a flotation revealed numerous eggs. A dilution count by the Stoll method gave 1,200 eggs per gram. At necropsy the gastric mucosa was covered with a catarrhal exudate in which numerous nematodes were visible. The mucosal surface exhibited areas of inflammation with petechial and ecchymotic hemorrhages. Nodules about 1.5 to 2 mm in diameter were scattered over the surface between the folds, with a few occurring on the folds.

The stomach contents and washings were pooled, and the worms in an aliquot portion were counted. Since many worms still adhered to the mucosa, the stomach was digested by the method of Herlich (1956, Proc. Helminth. Soc. Wash. **23**: 102–103) and the worms in an aliquot portion of the suspension were counted. The number of adult *H. rubidus* present in the stomach, thus estimated, was 11,200. In addition there were present 43,280 larvae, about 45% of which were fourth stage, 52% in the fourth molt, and 3% fifth stage.—F. G. TROMBA AND F. W. DOUVRES, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland.*

A REDESCRIPTION AND LIFE-HISTORY OF *GIGANTOCOTYLE EXPLANATUM* (CREPLIN, 1847) NASMARK, 1937 (TREMATODA: PARAMPHISTOMIDAE) FROM INDIA

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Gigantocotyle explanatum (Creplin, 1847) Nasmark, 1937 is a very common parasite of the domestic ruminants in India and is found in the liver, bile ducts and gall bladder, usually accompanying infection with *Fasciola*. In the adult stage the parasites are non-pathogenic, or only slightly so, but immature forms when present in large numbers cause amphistomiasis which in the majority of the cases proves fatal to the host. This amphistome has been reported from *Bos taurus indicus* and *Bubalis bubalis* from India, Burma, Ceylon, Indo-China and the Philippines (Nasmark, 1937; Dawes, 1946).

Except for a short and incomplete account given by Srivastava (1944) and a study of the structure of the miracidium by Ozaki (1951), no account of the life history of *Gigantocotyle explanatum* is available.

Since considerable difficulty was found in the identification of the parasite and there still seems to be some confusion, a short diagnosis of *Gigantocotyle explanatum* is included here for the sake of convenience and future reference. The redescription is based on a large number of specimens studied as whole mounts and serial sections, collected from buffalo at Lucknow and with which the present work was carried out. As regards the synonymy of the species, reference is made to Fischoeder (1903, 1904), Maplestone (1923), Travassos (1934), Dawes (1936) and Nasmark (1937). For the purposes of the present work *Gigantocotyle bathycotyle* (Fischoeder, 1901) Nasmark, 1937 is considered a synonym of *Gigantocotyle explanatum*.

MATERIALS AND METHODS

The adult amphistomes were obtained from buffalo slaughtered locally and a large number of gravid specimens were always available throughout the year. The gravid specimens were identified and the eggs obtained by teasing the uterus in a small amount of distilled water. When a large number of eggs had thus been collected from several specimens the eggs were freed from debris by decantation and kept in covered petri dishes. During the summer months the water of the dishes was changed twice daily but only once during the winter months. The eggs were kept at laboratory temperature during the summer months and in a constant temperature cabinet at 82° F ± 2° F during winter months.

A large amount of material was examined for the study of all the larval stages. Distilled water and 0.3% salt solution were used as mounting media. Usually the larval stages were examined in living condition without staining but the following *intra vitam* stains were also found useful: Neutral red, brilliant cresyl blue, methylene blue, methyl green and Bismarck brown. For making permanent preparations the material was fixed in warm alcoholic Bouin's fluid or Susa's fluid and stained with Ehrlich's haematoxylin, borax carmine, acetic acid alum carmine and Semichon's carmine. For sectioning, the material was fixed in alcoholic Bouin's fluid, cleared in cedarwood oil, embedded in wax and cut at 6–12 microns. The sections were stained with Ehrlich's haematoxylin and eosin, Heidenhain's iron haematoxylin and Mallory's triple stain. For the study of the cercaria, the ordinary methods gave poor results. Serum of sheep was used as a mountant for cercariae for studying the excretory system. For the study of the genital rudiments Schneider's acetocarmine was found to be an ideal stain and was used as described by Singh (1955). The permanent preparations were, however, not satisfactory.

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For the study of the ciliated epidermal plates, the miracidia were stained with methylene blue while alive. For making permanent preparations, hot 0.5% $AgNO_3$ was used to bring out the furrows between the plates. To ascertain the total number of plates some silver-impregnated miracidia mounted in balsam were slightly crushed, thus bringing all the plates almost to the same level.

The snails used in the infection experiments were reared either from eggs collected from nature or from the aquarium in which adult snails had been kept, and thus a regular supply of infection-free snails was always available.

Part of this work was done by me when employed in the Helminthiasis Scheme, I.C.A.R., University of Lucknow. My thanks are due to Prof. G. S. Thapar who has kindly helped me at all stages of this work, to the Indian Council of Agricultural Research for providing funds, and to the authorities of the University of Lucknow for laboratory facilities.

LIFE HISTORY

Egg: The eggs were kept in distilled water in petri dishes and the majority of them developed into miracidia. The eggs when deposited are full of light yellowish-green yolk cells which can readily be distinguished from the single transparent propagatory cell which is central in position. The eggs have thin colorless shells provided with operculums at the narrower ends. They measure 0.121–0.155 × 0.077–0.09 mm with an average size of 0.14 × 0.086 mm.

Miracidium: The miracidia hatch in about 9–16 days from the eggs, depending upon temperature, and almost all the eggs in one batch hatch within 72 hours after the first miracidium. The miracidia usually come out in the morning from 6–10 a.m. The time required for the complete development is shortened during the summer months and the miracidia may hatch out within 6 days. The effect of temperature on the development of miracidium has been studied and reported in short by Singh (1956). Both artificial light and sunlight have the effect of hastening and "touching off" the process of hatching, although eggs which have been kept in almost total darkness produce miracidia but require a little more time.

The miracidium is of the usual amphistome type and measures 0.15 mm in length and 0.067 mm in breadth at the shoulder. A single pair of penetration glands is present, 1 on each side. They are elongated and flask-shaped, measuring 0.03–0.032 mm in length with nuclei 0.005–0.006 mm in diameter. Ozaki (1951) also found 1 pair of glands in *Paramphistomum explanatum,* though usually 2 pairs have been described in other amphistomes. The nerve mass is present at the anterior end and is sometimes difficult to see. The apical gland (primitive gut) is present at the anterior end in between the penetration glands, and opens at the apex of the papilla. The 2 large flame cells are present almost in the middle of the body, somewhat close to the lateral margins. The excretory ducts run posteriorly from the flame cells and before reaching the posterior end of the body turn back forming a loop before opening on the lateral margins in the posterior half. The excretory pores are present at the junction of the third and the fourth row of ciliated epidermal plates. The posterior region of the miracidium produces the propagatory cells which form the germ balls. The 2 germ balls are composed of several large cells which develop into embryos and these are followed by a string of germ cells reaching to the posterior end. The nuclei of the germ balls are 0.004 mm in diameter. The number of germ balls varies from 1 to 3, usually 2 being present. The germ cells may be 12–23 in number.

The ciliated epidermal plates are present in 4 rows: the first row has 6 plates, the second row 8, the third row 4, and the fourth and last row 2 plates. In all

amphistomes where the epidermal plates have been counted they are present in rows as 6,8,4,2 = 20. Sinitsin (1931) described the epidermal plates of *Paramphistomum cervi* to be present in 5 rows as 6,6,3,4,2 = 21. Evidently, Sinitsin was working with a species other than *P. cervi*, probably not an amphistome at all. The nuclei of the plates are present near the posterior margins. They are elongated and measure 0.007–0.008 mm.

Intermediate host: For experimental infections a large number of snails of the following species were used: *Lymnaea acuminata, Indoplanorbis exustus, Melanoides tuberculata, Vivipara bengalensis, Bulinus* sp., *Amnicola* sp. and *Gyraulus convexiusculus*.* The snails were kept in large earthen troughs and were fed on lettuce leaves. The eggs of the snails were collected and transferred to the aquaria where they hatched. Snails of all ages were used for infection experiments and no attempt was made to find at what age the snails are most susceptible to infection, for it was observed in initial experiments that snails of all ages readily became infected.

A large number of snails of the various species were removed to a jar and to this were added a large number of actively swimming miracidia which had just emerged. After 6 hours the snails were sorted out according to their species and specimens of each species were kept separately in aquaria. After about 2 weeks each snail was isolated in a small amount of water in a tube and examined daily for the emerging cercariae. In 1 experiment, some cercariae were shed by *Gyraulus convexiusculus* 27 days after infection. On superficial examination the cercariae were found to be amphistome of the Pigmentata group. In other experiments the cercariae were shed from 25 to 64 days after the snails were exposed to miracidia. In 1 experiment the cercariae continued emerging up to 85 days when all the infected snails died.

A number of other experiments were conducted to infect other snails, specially *Indoplanorbis exustus* which was reported as an intermediate host for *G. explanatum*, but the results were always negative. In the case of *Gyraulus convexiusculus* the incidence of infection was always very high, very few snails escaping infection.

Sporocyst: The snails were examined for sporocysts 24 hours after they were exposed to miracidia but the youngest specimens of sporocysts recovered were 96 hours old.

The 4-day sporocyst is small, rounded or slightly elongated and shows little mobility. It consists of a thin-walled sac with a large cavity inside containing 4–8 germ balls. One sporocyst which was rounded measured 0.136 mm in diameter with 4 rounded germ balls present, the larger 2 of which were 0.065 mm and 0.034 mm in diameter. The other sporocyst was slightly elongated and measured 0.224 × 0.13 mm. It contained 2 germ balls, 0.065 mm and 0.047 mm in diameter, and 6 smaller germ balls were present at the posterior end. The maximum number of germ balls found in a sporocyst was 11. The epithelium of the sporocyst is thin and contains large nuclei. At the posterior end of the sporocyst are present several large cells, usually 3–5 in number and 0.011 mm in diameter. These are the germ cells of the sporocyst.

* The snails were identified by the Zoological Survey of India through the courtesy of its then director, the late Dr. S. L. Hora.

The wall of the sporocyst at this stage consists of a thin cuticle with numerous cells, the larger cells measuring 0.012–0.013 mm and the smaller cells 0.009–0.01 mm in diameter. The nuclei are large and prominent. The muscular layers are slightly developed and the sporocyst appears as a rounded bag with very thin walls. The wall is 0.002 mm in thickness. One pair of flame cells is present which open to the outside through short excretory ducts without any cilia.

On the sixth day, from one snail 3 well developed sporocysts and 2 immature rediae, and from another snail a single sporocyst, were recovered. One of the sporocysts at this stage showed 11 germ balls, the largest of which was elongated but did not show any recognizable organs of the redia. The other sporocyst, however, showed a more advanced stage of development as the largest germ ball had already assumed the shape of the redia showing the muscular pharynx and intestines. Besides this redia there were 9 other germ balls (embryos) in various stages of development. It appears that a sporocyst is fully developed in 6 days, as not only this sporocyst but an immature redia outside the body of the sporocyst was also recovered from the snail. Sporocysts from which 1 or more rediae have escaped are recovered up to the 12th day, though one sporocyst containing a few developing embryos was recovered from a snail after 28 days of infection.

The wall of the 6-day sporocyst is considerably thicker, measuring 0.015–0.016 mm in thickness. The majority of the larger cells, so numerous in the 4-day stage, have almost completely disappeared. The cuticle is thin and is followed by a layer of muscle fibers and subepithelial cells. The subepithelial cells are small, measuring 0.01 mm in diameter and possess small nuclei sparsely distributed. The muscle fibers are well developed.

In 1 experiment, the cercariae were shed by the snail 24 days, and the snail was dissected on the 27th day after exposure to miracidia. Besides a number of cercariae and rediae in various stages of development, 1 sporocyst was also recovered. The sporocyst possessed 7 germ balls in various stages of development, and one of these was elongated in shape but did not show any signs of pharynx or intestine of a redia. At 1 end there was an opening caused by the escape of the rediae. The muscles at the end were contracted like the strings of a purse.

Redia: The youngest redia recovered from a snail was 6 days old. This redia measured 0.236 mm in length and 0.074 mm in breadth. The muscular pharynx was 0.037 mm in diameter and the intestine 0.074 mm in length. It contained 9 germ balls of various sizes. The germ balls are composed of large cells with prominent nuclei. A small pear-shaped sac was present immediately posterior to the muscular pharynx, probably representing the rudimentary esophagus. Five elongated cells were observed in this region representing the esophageal gland cells. The cells lining the intestine are very big in size and the nuclei prominent.

The mature rediae measure 0.998–1.05 mm in length and 0.19–0.21 mm in breadth. The redia contains a muscular pharynx, a sac-like intestine and a birth pore near the anterior end. The cercariae in various stages of development are present inside the cavity. The excretory system consists of 3 flame cells on each side. The anterior pair of cells is present close to the intestine, the middle pair a little posterior to the excretory pore and the posterior pair in the posterior part of the body. The excretory pores are present in the posterior half, about ¾ from the anterior end. The redia does not possess any collar or lateral appendages. In

the younger rediae, 3 pairs of gland cells can be seen by the side of the intestine but in older stages the number of cells increases.

In 1 redia which was damaged at the posterior end and therefore incomplete, 16 cercariae in various stages of development were present. According to Looss (1896) daughter rediae and even third generation rediae are found in *Paramphistomum cervi*. Subsequently other authors reported several generations of rediae for amphistomes. Numerous snails were examined both in summer and winter months but not a single redia with daughter rediae was found.

In immature rediae the cuticle is thin and the subepithelial cells are 0.01 mm in diameter and possess large nuclei. The muscular layer is slightly developed and all the subepithelial cells are of the same size. The cells of the intestine are large. The birth pore is present though not conspicuous. In mature rediae, the cuticle is somewhat thicker and the subepithelial cells vary in size, being 0.01–0.022 mm in diameter. The large and smaller cells are unevenly distributed. The muscular layers, especially the circular muscles, are well developed. The wall of the intestine becomes thin at places. The germ cells which give rise to the germ balls are present at the posterior end and measure 0.015 mm in diameter. The body wall is 0.012–0.013 mm thick.

Cercaria: The cercariae are liberated from the redia while they are still immature and complete their development outside the body of the redia and the snail. As stated above the period required for the shedding of the cercariae, from the day the snails have been exposed to miracidia, varies considerably.

The cercariae are shed in the morning and continue to emerge for 2–3 hours every day for as many as 20 days. The free-swimming life of the cercaria is usually short, ranging from 20 to 60 minutes, average, 40 minutes.

The cercariae are easily seen with the naked eye due to their size, dark pigmentation and active swimming. The cercariae were measured at rest without any pressure and the following are the average measurements: Length of body, 0.496 mm; breadth of body, 0.341 mm; length of tail, 0.62; breadth of tail, 0.062 mm; diameter of ventral sucker, 0.155 mm.

The body is heavily pigmented on the dorsal side and sparsely so on the ventral side. The pigmentation starts as a ring around the eye spots in the immature cercaria. This pigmented area soon assumes stellar shape and sends narrow "strings" of pigmentation on all sides, especially towards the posterior side. These "strings" form a net-work and gradually the meshes assume a heavier pigmentation until the whole body is closely covered by stellate areas of very dark brown pigment.

The 2 eye spots are present in the anterior region on the dorsal side and appear as posteriorly directed pigmented cones with clear lenses. The body is full of rounded cells containing rod-like cystogenous matter. The digestive system consists of an oral sucker, 0.062 mm in diameter followed by an elongated esophagus. A pharynx is absent. Just posterior to the base of the eye spots, the esophagus divides into 2 intestinal ceca which extend almost up to the posterior margin of the posterior sucker (acetabulum). The rudiments of the genital organs can be made out only after staining with aceto-carmine. They consist of an oval mass of cells immediately anterior to the ventral sucker representing the ovary, a string of cells running anteriorly from the ovary representing the uterus, and 2 rounded masses of cells, 1 on each side of the uterus, representing the anterior and posterior

testis. The left testis is posterior to the right testis but both are of equal size. Two narrow strings of cells representing the vasa efferentia run anteriorly from the 2 testes. The genital pore is represented by a mass of cells, somewhat sausage-shaped, present at the intestinal bifurcation but always anterior to it.

The excretory system is difficult to trace due to the presence of a large amount of cystogenous matter and the pigment. The rectangular excretory vesicle is present immediately anterior to the posterior sucker. The 2 main excretory ducts open at the posterior end of the excretory vesicle through a common opening. The main excretory ducts run forward for a short distance and, almost in the midregion of the body turn medially to join the duct of the other side, thus forming a transverse connection. A small blind duct runs forward from the transverse duct. From the level of the transverse duct, the main excretory ducts run forward and outward, and just posterior to the eye spots divide into 2 ducts—1 duct running on the medial side of the eye spot up to the anterior end and the other small duct running externally to the eye spot. Bennett (1936) observed that this invagination of the excretory duct is present in *Paramphistomum microbothrioides* (= *C. cotylophorum*), but absent in *Paramphistomum cervi*. Bennett probably based this distinction on the description of the cercaria as given by Looss (1896). Brumpt (1936) evidently observed this invagination. The posterior sucker is provided with a network of small ducts. I could detect only 2 small ciliated areas in the course of the ducts of the posterior sucker. The caudal excretory duct arises from the excretory vesicle and runs as a wide duct through the tail. At the posterior end of the tail, the duct enlarges slightly and gives off 2 narrow ducts opening to the outside laterally. The excretory pore is present on the dorsal side of the excretory vesicle. The main excretory ducts, the transverse excretory duct and the 2 anterior ducts possess refractile excretory granules of various sizes. The development of the excretory system in the larval stages has been studied and forms the basis of another paper (Singh, 1957).

Encystment: After the cercaria has been swimming for some time it attaches itself to the wall of the container or to leaves, almost invariably near the water level. In case the cercaria attaches much below the water level or drops down to the bottom, it creeps up till it reaches this level. Shortly, the cercaria stops body movements but the tail keeps on lashing from side to side. The cystogenous material comes out of the body and is distributed evenly all around it due to the rapid movement of the body in a semicircle and to the rapid lashing of the tail. Gradually the cyst is formed around the body and the tail is cast off, though both the body and the tail keep on moving for some time more. The cysts are dark brown in color, rounded in shape and can easily be seen with the naked eye. It was observed that when the encystment was allowed to proceed undisturbed, the cercariae always encyst close together. The cysts measure 0.2–0.23 mm in diameter. The wall of the cyst is fairly thick and hard, and 0.014–0.019 mm in thickness. The metacercariae when viewed through the cyst wall show the pigmentation and the refractile excretory granules only.

Infection of final host: For infecting the final host a goat kid which had been reared in the laboratory since birth was used. About 350 metacercariae which had encysted on lettuce leaves were fed orally to the kid over a period of 6 weeks and the kid was examined approximately 16 weeks after the first and 11 weeks after

the last feeding of metacercariae. Both mature and immature specimens of *G. explanatum* were recovered. This indicated that parasites reach maturity in less than 16 weeks after the metacercariae enter the host.

DISCUSSION

Sonsino in 1892 described from various snails from Egypt an amphistome cercaria which is now well known as *Cercaria pigmentata*, but the account was incomplete. The same cercaria was described by Looss (1896) in detail and Looss affirmed that the cercaria belonged to *Amphistoma conicum* (= *P. cervi*). Looss found more than 2 generations of rediae and counted at least 5 pairs of flame cells as compared to 1 generation of rediae and 3 pairs of flame cells found in the present species. In *Cercaria pigmentata*, as described by Looss, the excretory duct does not give off a small duct or form a coil near the eye spot. In the present material as well as in *P. microbothrioides* as described by Bennett (1936), the coil is present near the eye spots. Though Looss has neither described nor labelled the rudiments of testes in his diagram of *Cercaria pigmentata*, he has shown 2 rounded masses of cells, 1 on either side and on the same level, which presumably are the rudiments of testes. A very large number of cercariae and immature specimens were examined in the present material, but in all cases the testes were found one behind the other. Since the 2 testes in the adult *G. explanatum* are present one behind the other or diagonally so, it is only reasonable to expect that they will be so in the cercarial and immature stages. It is true that during development some of the organs do change their positions slightly but this can always be followed by intermediate stages. Consequently, such radical changes, which must be presumed if Looss' work is accepted to be correct, are rather strange. It appears to me that Looss was not working with the larval stages of *P. cervi*.

Sewell (1922) described some new amphistome cercariae from India and placed them in Pigmentata group along with *Cercaria pigmentata*. One of these cercariae, *Cercariae indicae XXVI* recovered from *Indoplanorbis exustus* closely resembles the cercaria described above. In *Cercaria indicae XXVI* there is only 1 generation of rediae and it possesses only 3 pairs of flame cells. The 2 cercariae have similar excretory systems and the rudiments of testes are present one behind the other. They can be separated by the different disposition of the genital organs. In *Cercariae indicae XXVI* the testes are shown to be one behind the other but they are present on the left side of the ovary and the uterus, and also, the posterior testis is present at the level of the ovary and both the testes are posterior to the dorsal excretory pore. Chatterji (1931) recovered some cercariae from naturally infected *Indoplanorbis exustus* at Rangoon and identified them as *Cercariae indicae XXVI* Sewell, 1922. Chatterji, like Sewell (1922) considered the cercaria to be different from *C. pigmentata* and *C. frondosa* Cawston, 1918, respectively belonging to *P. cervi* and *P. explanatum*, and he expected *C. indicae XXVI* to develop into some other species of *Paramphistomum*. Rao & Ayyar (1932) recovered some cercariae from naturally infected *Indoplanorbis exustus* at Madras. They identified the cercariae as *C. indicae XXVI* and by feeding the metacercariae to a calt they recovered amphistomes which they identified as *P. cervi*.

In 1936 Brumpt recovered amphistome cercariae from naturally infected *Bulinus contortus* and by feeding these to sheep and goat he recovered *P. cervi*. The de-

tails of the various larval stages are unfortunately lacking but presumably there is 1 generation of rediae present. From the figures provided, the lateral extension of the excretory duct can be made out near the eye spot but the median duct connecting the 2 lateral ducts is missing. In the former condition Brumpt's material agrees with *Cercariae indicae XXVI* Sewell, 1922 and the present material, but in the latter character it agrees with Looss' and Sonsino's description of *Cercaria pigmentata.*

In the same year Szidat (1936) also worked on the larval stages of *P. cervi* but did not give full details. The excretory system as shown by him in his figure 3b, p. 11, differs from the system in the present material in the absence of the lateral extension near the eye spot, and the median extension. In this character Szidat's material agrees with Brumpt's material.

Porter (1938) recovered cercariae from naturally infected *Bulinus tropicus* and *Lymnaea natalensis* from South Africa and identified them as those of *P. cervi* even though she could see only 3 pairs of flame cells in the redia. She found the rudiments of the testes present one on either side of the ovary and level with it, which is rather unexpected since the testes in *P. cervi* are present one behind the other and both of them are present anterior to the ovary. Since she did not perform any feeding experiments and was working with naturally infected material, it seems more probable that she was working with some amphistome other than *P. cervi.* She also described *Cercaria paramphistomi calicophorum* from *Bulinus tropicus* from South Africa. The redia differs from the present material in the possession of lateral appendages. The cercaria also differs in the possession of esophageal outpockets, the position of the testes and the size of the excretory bladder. Further, the adults that she obtained after feeding experiments had the testes side by side.

Dawes (1936, p. 346) suggested that *P. explanatum* and *P. gigantocotyle* may be synonyms of *P. cervi,* the decision to be reached on the evidence of the life history. Grobbelaar (1922) and Le Roux (1930) consider *Cercaria frondosa* Cawston as belonging to *P. calicophorum* [synonym of *P. explanatum* according to Maplestone (1923) and of *Calicophoron calicophorum* according to Nasmark (1937)] and *Cotylophoron cotylophorum* respectively. But as Bennett (1936) points out, and I agree with him, *Cercaria frondosa* Cawston as described by Faust (1919) could not possibly belong to either of them. Srivastava (1944) claimed *Indoplanorbis exustus* to be the intermediate host of *P. explanatum* in India but the full paper has not appeared. Since I have repeatedly failed to infect *Indoplanorbis exustus* with the miracidia of *Gigantocotyle explanatum,* it seems that *Calicophoron calicophorum, Gigantocotyle explanatum* and *Paramphistomum cervi* are distinct species after all.

Peter & Mudaliar (1948) described a cercaria from Madras which they considered to be new and belonging to *Gastrodiscus secundus.* Anantraman & Balasubramaniam (1949) considered the above cercaria to be the same as *Cercaria fraseri* Buckley, 1939 whose adult is still unknown, and they also came to the conclusion that *Cercariae indicae XXVI* Sewell, 1922 is the proved larva of *Cotylophoron cotylophorum.* It is difficult to come to a conclusion since they have omitted to give most of the details and other workers who have worked on the life history of *Cotylophoron cotylophorum,* like Bennett (1936), Srivastava (1938), and Sinha (1950), have considered the 2 cercariae to be different.

Durie (1953) described the life history of *Ceylonocotyle streptocoelium* and *Paramphistomum ichikawai* from Australia. The larval stages of these amphistomes differ from those of *G. explanatum*. The miracidia of these amphistomes possess 2 pairs of penetration glands whereas the miracidium of *G. explanatum* possesses only 1 pair. Structurally the rediae of all 3 species are similar, but in *G. explanatum* only 1 generation of rediae is found while the other 2 have more than 1 generation. The cercaria of *G. explanatum* comes nearer to that of *P. ichikawai* rather than to *C. streptocoelium*, specially in the pattern of the excretory system. The two, however, differ in the size of the body, pharynx and acetabulum and the organization of the rudiments of the genital organs. In the interpretation of the latter, I believe, Durie was mistaken in considering the anterior mass to be representing the 2 testes, and the posterior mass the ovary. Staining the cercariae with acetocarmine will probably show a similar arrangement as described above for *G. explanatum*.

Dinnik & Dinnik (1954) described the life history of *P. microbothrium* from East Africa and *Bulinus aluaudi* to be the intermediate host. They found the first generation of rediae producing daughter rediae and cercariae together and usually 4 generations of rediae were produced. They also agreed with Nasmark (1937) that Looss' *A. conicum* could be *P. microbothrium*.

Redescription of *Gigantocotyle explanatum*
(All measurements in millimeters)

The following redescription is based on a large number of specimens collected alive. Usually a large number of specimens, ranging up to 100 or more, were found in the liver, although Nasmark (1937, p. 473) limits the number to 24, usually less.

Size: Varies considerably. Specimens fixed under pressure 14.54–16.62 (15.24)* long and 5.77–6.69 (6.23) wide. Specimens fixed without pressure 10.12–12.21 (11.17) long and 3.6–4.3 (3.95) wide in acetabular region (acetabulum excepted). For sectioning, gravid but smaller worms, 4.2–5.57 long were used. Dorsoventrally the worms measure 3.25–3.8 (3.54) in unflattened specimens.

Shape: Conical due to comparatively large size of acetabulum. Dorsal line always curved, though varying in degree.

Pharynx: Explanatum type, 0.6–0.76 (0.71) in length, ratio between pharynx and body length 1 : 6.4–7.95 (7.02); in sections elliptical, the 2 ends coming close to each other. Papillae absent. *Musculature:* Internal circular muscles fairly well developed, running along length of body. Internal longitudinal layer evenly developed except in anterior region. Middle circular layer well developed and easily distinguishable, several bundles placed side by side. Anterior sphincter muscles usually in continuation with bundles or separate. Radial layer with several fibres, evenly developed. External circular layer present at anterior end only, about 15 units. External longitudinal layer with several fibres. Basal circular layer well developed, 12–14 units. Posterior sphincter not as clear and distinct as shown by Nasmark but more like that of *G. gigantocotyle*. Anterior sphincter fairly well developed, 9 units, rarely 2 units present side by side as in *Pisum* type. Lip sphincter absent. Esophagus 0.6–0.61 long, divides into 2 intestinal ceca which run up to the anterior margin of acetabulum.

Male genital organs: Testes one behind the other, usually rounded even when closely applied, lobation may not be apparent in whole mounts. Testes vary in size, shape and lobation. In sagittal sections testes 0.66–0.95 (0.81) in length and 1.11–1.54 (1.29) in dorsoventral direction; in whole mounts 2–2.46 by 3.1–3.9. Posterior testis may be smaller or equal to anterior testis. Vasa efferentia join anterior to anterior testis and open to the outside posterior to intestinal bifurcation. Cirrus sac absent. Position of genital pore varies with age and stage: in cercaria, anterior to intestinal bifurcation and in mature specimens, posterior to bifurcation. Genital atrium of *Explanatum* type. Genital papilla sometimes projects beyond body line and consequently pore quite superficial, but in others, at the base of a short and shallow depression while in one specimen, a definite narrow canal present but not deep.

Female genital organs: Ovary at posterior end, on right side. Mehlis' gland on medial

* Measurements in parentheses refer to average of several specimens.

side of ovary. Vitelline follicles large, mainly on lateral sides, from near esophagus up to the anterior region of acetabulum. Uterus runs anteriorly, medial and dorsal to testes, convoluted at terminal end; metraterm absent. Laurer's canal crosses excretory duct and opens posterior to it. Eggs 0.121–0.155 (0.14) by 0.077–0.09 (0.086).

Acetabulum: Explanatum type with elliptical or oval opening directed ventrally. Dorsal external layer I with 17–24 (20) units, well marked off from layer II which has 32–36 (34) units. Dorsal interior layer with 38–43 (41) units. Ventral interior layer with 40–59 (50) units. Ventral external circular layer with 12–18 (16) units. Radial musculature evenly developed. Acetabulum measures 2.06–2.58 (2.36) in diameter in sagittal sections of specimens 4.2–5.57 long. Ratio of acetabulum to body length 1 : 1.7–2.32 (2.12). In specimens 10.12–12.21 (11.17) long, acetabulum is 4.5–5.45 (4.96) in diameter with a ratio of 1 : 2.06–2.58 (2.25). In specimens fixed under pressure, acetabulum is 4.48–4.7 in diameter in specimens 11.71–13.83 long with a ratio of 1 : 2.59–3.08 (2.82).

Specimens measured by Nasmark were 8.48 in length but in literature the length given is 8–13. That the large number of parasites bring about reduction in size is well known (Rankin, 1937; Willey, 1941; Dawes, 1946), but here apparently the large number present does not seem to affect the size. The genital atrium of the present material agrees more with the *pisum* type in having a muscular, well developed and clumsy papilla and a shallow genital atrium but differs from it in the absence of a ventral atrium. In one specimen a definite narrow canal was present but even then it was not deep (see Nasmark figs. 81, 82).

Because of the rounded testes present one behind the other in several specimens, it was sometimes difficult to identify the material. Nasmark was doubtful about the validity of *G. bathycotyle* as a distinct species from *G. explanatum* but Willmott (1950) considered them to be distinct though assigned no reasons for it. She had obtained the material from rumen of buffalo (*Bos indicus*) from Ceylon. She described 2 testes lying one behind the other, the posterior one being wedge-shaped, and both slightly lobed and smooth in outline. The present material agrees with the above characters of testes, but with the data given by her I am unable to identify her material with mine. Nasmark (p. 478) gave 6 possible differences between these 2 species. In size, the present material is smaller than both the species as described by Nasmark. The acetabulum is comparatively large in *G. explanatum* with an oval or elliptical opening and the present material agrees with it. In my opinion the position and lobation of testes is a very variable character and hence of no specific value, at least in this genus. The relative size of pharynx is smaller in *G. explanatum* and it is even smaller in the present material, the ratio being 1 :6.4–7.95 with an average of 1 :7.02. The thickness of integument will largely depend on the mode of fixation, and will be a difficult character to compare. Consequently, the present material was identified as *G. explanatum* as accepted by Nasmark and *G. bathycotyle* is considered a synonym.

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Explanation of Plates

Figures 1–23. *Gigantocotyle explanatum.* All figures drawn with the aid of a camera lucida. 1. Egg, from uterus. 2. Egg, embryo partially developed. 3. Egg, with miracidium. 4. Miracidium, alive. 5. Miracidium, showing ciliated epidermal plates. 6. Miracidium, stained with Ehrlich's haematoxylin. 7. Sporocyst, early stage. 8. Sporocyst, late stage. 9. Sporocyst, 28 days old. 10. Redia, free early stage. 11. Redia, later stage. 12. Cercaria, free within the shell of snail, early stage. 13. Cercaria, free within the shell of snail, later stage. 14. Cercaria, free swimming. 15. Metacercaria, on leaf. 16. Cercaria, free, stained with aceto-carmine. 17. Immature adult from final host. 18. Immature, adult from final host. 19. Gravid adult from final host, ventral view. 20. Adult, sagittal section of posterior half. 21. Adult, sagittal section of pharynx. 22. Adult, sagittal section of acetabulum. 23. Adult, sagittal section of genital papilla and atrium.

Abbreviations Used

acet.—acetabulum
b.p.—birth pore
cerc.—cercaria
cil.—cilia
cil.pl.—ciliated epidermal plate
cy.c.—cystogenous cells
cy.w.—cyst wall
emb.—embryo
e.sp.—eye spot
ex.bl.—excretory bladder
ex.d.—excretory duct
ex.p.—excretory pore
fl.c.—flame cell
g.b.—germinal ball
g.c.—germ cell
g.p.—genital pore
gr.—groove
g.ru.—genital rudiments
Int.—intestinal caecum, intestine
met.—metacercaria
m.gl.—Mehlis' gland
mir.—miracidium
m.o.—mouth opening
nu.—nucleus
oes.—oesophagus
op.—opening
oper.—operculum
op.L.c.—opening of Laurer's canal
ov.—ovary
p.gl.—penetration glands
phar.—pharynx
pigm.—pigment
red.—redia
t.—tail
t_a.—testis, anterior
t_p.—testis, posterior
ut.—uterus
v.def.—vas deferens
vit.—vitellaria
vit.d.—vitelline duct

RESEARCH NOTE

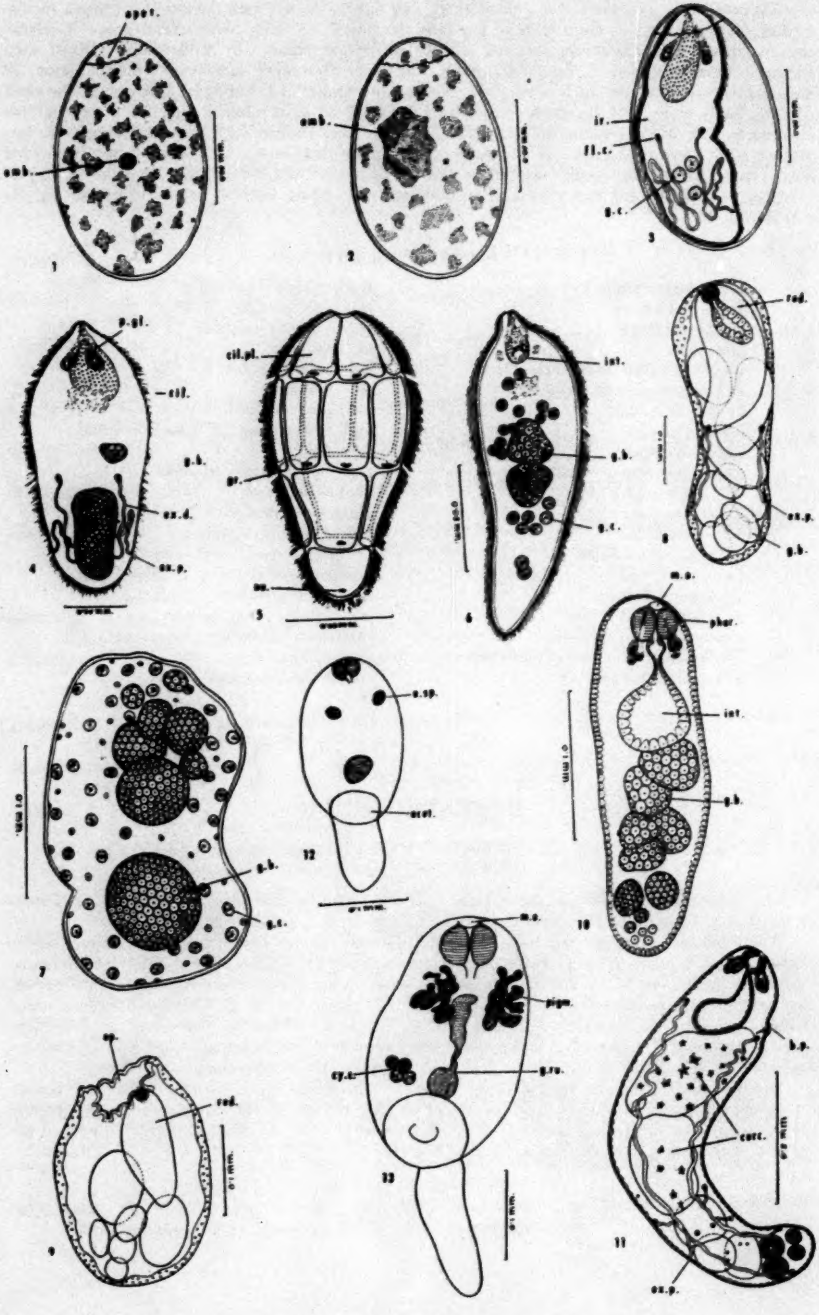
AN INSTANCE OF ERRATIC PARASITISM IN THE SKUNK, *MEPHITIS MEPHITIS*

On September 13, 1957, a skunk head was submitted to the animal diagnostic laboratory of the North Dakota Agricultural Experiment Station as a rabies suspect specimen.

The head had been severed from the body, without further examination, by a veterinarian approximately 6 hours prior to its receipt. Upon opening the brain cavity 2 adult female nematodes were found, one in the otic capsule and the other in the cavity overlying the cortical region. The specimens were identified by K. C. Kates, J. T. Lucker and M. B. Chitwood of the Animal Disease and Parasite Research Division, A.R.S., U.S.D.A., Beltsville, Maryland, as *Physaloptera* sp., probably *P. maxillaris* Molin, 1860, the common stomach nematode of skunks, and are deposited as No. 55974 in the U. S. National Museum, Helminthological Collection.

Smears made from the hippocampus region of the brain were positive for Negri bodies. Encapsulated nematode larvae were observed in this region of the brain presumably having been deposited by the female worms.—R. F. Shumard and F. M. Bolin, *North Dakota Agricultural Experiment Station, Fargo, North Dakota.*

PLATE I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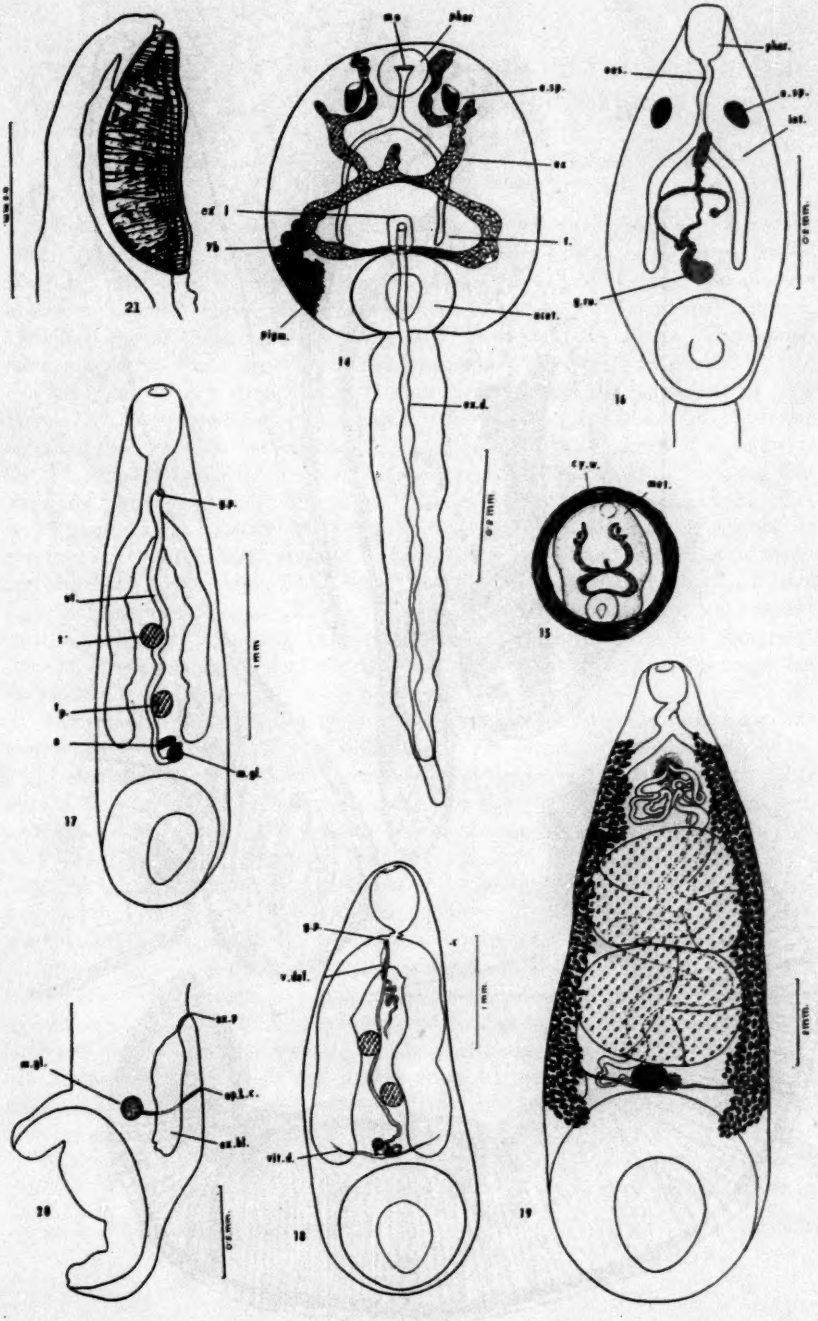
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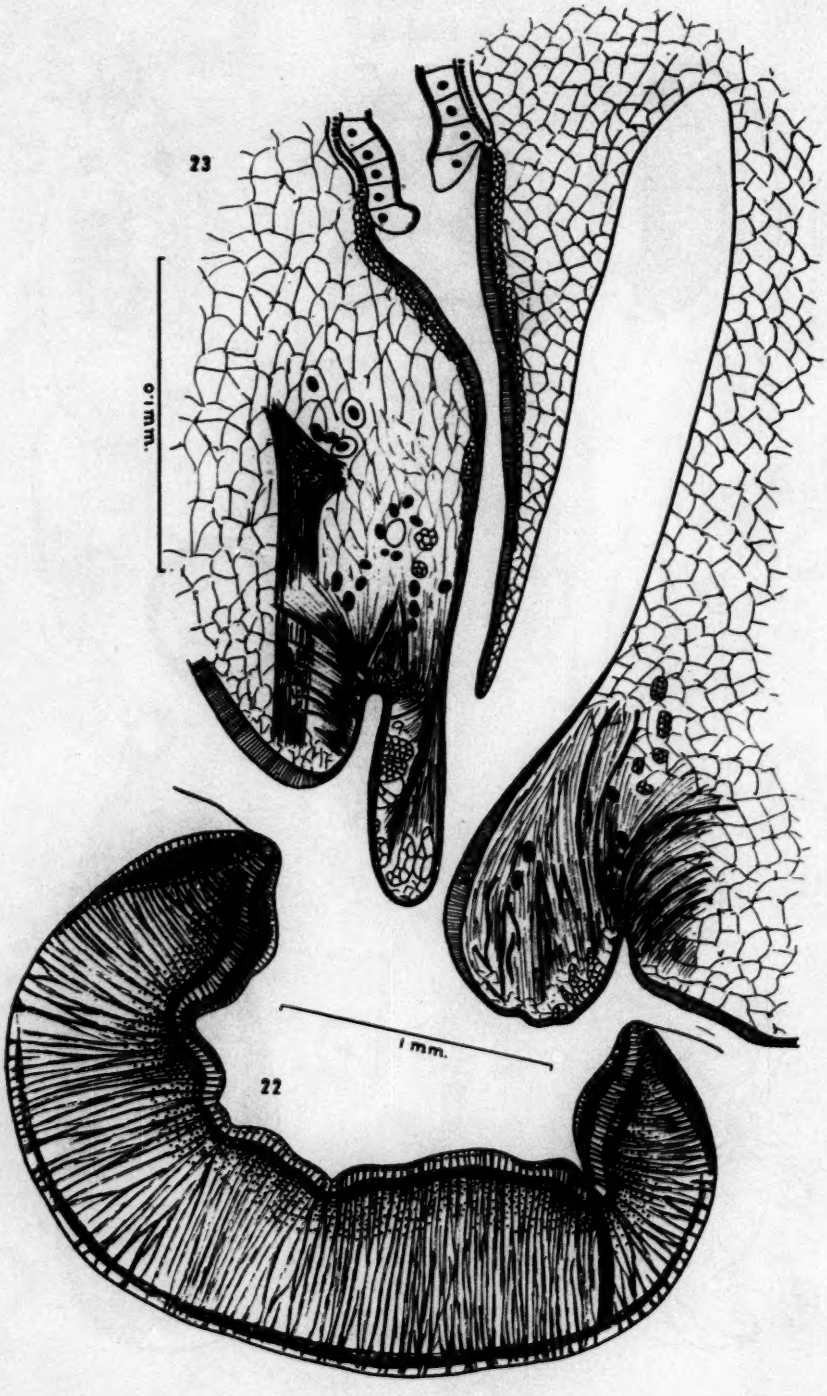
PLATE III



THE MORPHOLOGY AND LIFE-HISTORY OF *LEVINSENIELLA MINUTA* (TREMATODA: MICROPHALLIDAE)

HORACE W. STUNKARD

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Price (1934) described *Levinseniella minuta* from the small intestine of the scaup duck, *Nyroca affinis*, taken at Flamingo Lake, Culebra Island, in the West Indies. He noted that it is the smallest species of the genus and perhaps the smallest known trematode. Three specimens were deposited in the U. S. National Museum, Helminthological Collection; type, No. 8703; paratypes, No. 8704. The species has not been reported since the original, preliminary description by Price.

One of my former students, Dr. Wilbur L. Bullock of the University of New Hampshire, in November, 1956 sent several hundred specimens of *Hydrobia minuta* (syn. *Paludestrina minuta*) collected near Durham, New Hampshire to the American Museum for study and identification of the 2 types of cercariae that were emerging from them. One of the cercariae was an heterophyid and the other was a microphallid. Dissection of the snails yielded a third trematode species, an encysted microphallid metacercariae. About 5% of the snails harbored these metacercariae, and the infection when present was intense, with hundreds of encysted metacercariae in each snail. In every instance, the tissues of the host were almost entirely atrophied, with mere vestiges of normal structures. The fact that there were no light or medium infections suggested that the cercariae do not emerge, but encyst in the snail where they were produced.

The encysted metacercariae from 4 snails were fed to a white mouse and the animal was killed 24 hours later. More than 100 worms, some with eggs in the initial portion of the uterus, were recovered from the intestine. Subsequently other mice were fed encysted metacercariae and killed after 48 and 72 hours. These animals yielded large numbers of sexually mature worms, but a mouse allowed to go 8 days was free of the infection. It is apparent that the worms do not persist for a long time in mice. The size and structure of the mature worms recalled the description of Price, and comparison with the specimens deposited by him in the Helminthological Collection disclosed complete agreement.

Eggs from worms developed in the mouse were fed to *Hydrobia minuta* and every one of 12 specimens contained encysted metacercariae when dissected 2 months later. No cercariae were observed in the dishes where the snails were kept and presumably none was shed. The snails were small ones, less than half-grown, but since they were not laboratory-raised, previous exposure to eggs of the parasite was not precluded. However, the 100% infection supports the probability of experimental infection.

At the Marine Biological Laboratory, Woods Hole, Massachusetts, on May 9, 1957, Dr. Donald M. Wootton found large numbers of encysted metacercariae in

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specimens of *Amnicola limosa* collected in cranberry ditches off the Quashnet River below Route 151, on Cape Cod. In these instances also, there were no mild infections of *A. limosa;* either no metacercariae or hundreds of them. The metacercariae agreed in size and general appearance with those of *Levinseniella minuta* and Dr. Wootton fed cysts from these snails to a half-grown hamster. Hundreds of mature specimens were taken from the intestine of the hamster 48 hours later. These worms were apparently identical with the ones developed in the intestines of white mice from metacercariae found in *H. minuta* from New Hampshire.

Metacercariae from *A. limosa*, provided by Dr. Wootton, were fed by the writer to a white mouse and hundreds of sexually mature worms were obtained 3 days later. To check on specificity, eggs from these worms were fed to specimens of *H. minuta* that had been kept in the laboratory since the previous winter. Every snail of 10 exposed showed infection when dissected and all stages from sporocysts to cercariae and metacercariae were obtained.

Description of Stages in the Life-Cycle

Adult (Fig. 6)

From dorsal or ventral aspect the worms vary in shape from ovate to pyriform to triangular; typically they are wider posteriorly although in movement either end may be wider. The body is curved, concave ventrally, and increasingly thick posteriorly. Although variation is less in fixed and stained specimens and mounted ones are slightly smaller than when alive, measurements were taken on living worms. Sexually mature specimens measure 0.12–0.20 mm long and 0.09–0.12 mm wide. The cuticula bears relatively large, closely set spines, which are somewhat smaller and more separated on the posterior half of the body. The acetabulum is situated about 2/3rds of the body length from the anterior end and measures 0.020–0.028 mm in diameter. The oral opening is subterminal, the sucker 0.023–0.030 mm in diameter. The prepharynx is very short and may completely disappear; the pharynx is 0.010–0.018 mm in diameter. Usually it is longer than wide, especially if the anterior end of the body is extended. The esophagus varies much in length, from 0.016–0.040 mm, as the anterior portion of the body is protruded and retracted. The ceca diverge slightly as they pass backward and then follow the lateral contours of the body. They may be slender or saccate and may extend backward to the level of the posterior edge of the acetabulum. The excretory vesicle is V-shaped and primary collecting ducts pass forward on each side to the middle of the body where they receive secondary ducts. On each side a secondary duct passes forward, dividing to form 2 tertiary ducts, and each tertiary receives the capillaries from 2 flame cells. A similar but obverse pattern obtains in the case of the other secondary duct which passes backward and receives the excretory wastes from the posterior half of the body. The flame-cell formula is $2[(2+2)+(2+2)]$, and the disposition of flame-cells and capillaries is virtually identical with that of *Microphallus similis* as figured by Stunkard (1957). On each side there are 2 flame-cells in the oral-pharyngeal region, 2 in the preacetabular region, 2 in the pretesticular and 2 in the posttesticular area.

The testes are oval, opposite, situated laterally and somwhat diagonally in the postacetabular region with their anterior ends pointing slightly laterad; they measure 0.012–0.029 mm in diameter. Sperm ducts pass forward and mediad and

unite to form a seminal vesicle, oval to reniform to semilunar in shape, 0.014–0.020 mm in length, situated medially or slightly on the ovarian side. It is preacetabular, extends from right to left, and a narrow duct, enclosed by a few glandular cells, leads to a small papilla which opens on the dorsal face of the genital atrium. The genital atrium lies just left of the acetabulum and the apposed faces are often somewhat flattened. The atrial cavity is 0.02–0.035 mm in diameter; its wall is folded, (Fig. 6), but there are no definite pockets and no cuticular supports, hooks or ribs. The wall is fibrous, without nuclei, and can be everted, forming a large, curved, club-shaped copulatory organ, about 0.018 mm wide at the base and 0.040–0.060 mm long, with the muscular papilla at its tip. The ovary is dextral, at the acetabular level; transversely oval, 0.015–0.020 by 0.021–0.024 mm. The vitellaria consist of 5 or 6 indistinct follicles, posterior to the testes and dorsal to the uterus; their ducts pass mediad and anteriad, joining to form a common reservoir which discharges into the ootype. Laurer's canal, probably present, was not observed. The ootype is located at the postero-median edge of the ovary, in the area between ovary and acetabulum. The uterus passes backward, forms a series of coils on the right side of the body and then crosses to the left side where a similar series of coils leads to the short metraterm which opens into the postero-median side of the genital atrium. The uterus is entirely postacetabular although coils may sometimes extend forward to touch the ends of the digestive ceca. The eggs are relatively large, 10 to 30 in number, and measure 0.020–0.022 by 0.012–0.013 mm. They are operculate and on deposition contain well-formed miracidia.

Sporocysts (Figs. 1, 2)

There are 2 sporocyst generations, since small daughter sporocysts were numerous in snails 2 months after infection. Small sporocysts are almost spherical, whereas older ones become oval to elongate. The larger ones have cercarial embryos together with germballs and germinal cells; they range up to 0.14 mm in length and 0.08 mm in width. The recognizable cercariae in a sporocyst are always few, seldom more than 3. Indeed, the total number of cercariae produced is very small, since all become metacercariae in the body of a single, minute snail.

Cercaria (Fig. 4)

The cercariae emerge from the sporocysts in a relatively immature condition. They are small, 0.07–0.095 mm in length and 0.050–0.060 mm in width, tailless, and without cuticular spines. Since they are produced in small numbers and encyst soon after emergence, the number available for study was limited. There is no acetabulum and the only obvious features of the cercaria are the oral sucker and the excretory vesicle. The flame-cell pattern of the cercaria was not worked out.

Metacercaria (Figs. 3, 5)

After encystment the cercariae continue to grow and mature. The cyst-wall is clear, almost homogeneous, and 0.008–0.010 mm in thickness. The diameter of a mature cyst may reach 0.10 mm, but the worms are infective at a smaller size. Worms from smaller and younger cysts require slightly longer to reach maturity in the final host, and become gravid at a slightly smaller size.

DISCUSSION

The genus *Levinseniella* was reviewed by Rankin (1939) who gave a detailed diagnosis and recognized 8 valid species. The diagnosis was based largely on the sexual generation, since at the time no complete life-cycles were known. The first life-history, with descriptions of larval stages, was done by Etges (1953) on a new species, *Levinseniella amnicolae*, whose asexual generations were discovered in *Amnicola pilsbryi* in New York. In the species described by Etges, the cercariae are of the characteristic Ubiquita type, have tails, are shed, and encyst in *Asellus communis*. The metacercariae developed to sexual maturity in mallard and domestic ducks, day-old chicks and white mice. They occupied the ceca, but not the intestines of ducks and mice. It is noteworthy that in *L. minuta* the life-cycle is abbreviated by encystment of the cercariae in the first intermediate host and the omission of a second intermediate host. Correlated with this modification of the life-cycle, the cercariae are tailless and emerge from the sporocysts at a relatively immature stage of development.

Scaup and other diving ducks feed largely on mollusks and eat snails, especially in late summer when the water level in ponds and streams is low. This habit makes them particularly susceptible to infection by parasites which use mollusks as second intermediate or paratenic hosts. Certain features of the life-cycle of *L. minuta* favor the maintenance and perpetuation of the species. Emergence of the miracidia only after ingestion of the eggs by a suitable host has survival value, since the larvae may remain viable in the eggs for long periods of time. Encystment of the cercariae in the snail where they developed avoids losses by death that would be certain to occur if the cercariae emerged, and every cercaria becomes a metacercaria in a paratenic or transfer host that is likely to be eaten by the final host. Thus *L. minuta* is able to maintain the species with the production of a relatively small number of cercariae. A similar situation occurs in *Typhlocoelum cymbium*, a parasite of the pied-billed grebe, reported by Stunkard (1934), where the cercariae encyst in the snail which serves as the first intermediate host.

ACKNOWLEDGMENT

Grateful appreciation is here expressed to Drs. Wilbur L. Bullock and Donald M. Wootton for material used in this investigation.

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EXPLANATION OF PLATE

Developmental stages of *L. minuta;* Figure 4 from a slightly compressed living larva, other figures from fixed and stained specimens.

FIGURE 1. Tip of abdomen of *Hydrobia minuta,* experimental infection, showing daughter sporocysts and developing cercariae.

FIGURE 2. Sporocyst, 0.078 mm long, with germ balls, from a snail other than the one shown in Figures 1 and 3.

FIGURE 3. Portion of abdomen of snail shown in Figure 1, compressed laterally, with an unencysted cercaria in the upper right corner, between lobes of the digestive gland, and many encysted metacercariae; same scale as Figure 1; dorso-ventral measurement, 0.75 mm.

FIGURE 4. Cercaria, 0.086 mm long, from haemal sinus of *H. minuta,* showing oral sucker, pharynx, excretory vesicle, and cell aggregates which are incipient stages of the acetabulum and gonads.

FIGURE 5. Encysted metacercaria, cyst 0.09 mm in diameter, from *H. minuta,* experimental infection.

FIGURE 6. Adult specimen, 0.17 mm long, from a white mouse.

RESEARCH NOTE

EXPERIMENTAL INFECTION WITH STRIGEOID CERCARIAE

During the summers of 1947 and 1948 strigeoid cercariae from Carrol Lake, Wisconsin were collected and identified by the late Dr. F. G. Brooks and turned over to the writer for use in the experimental infection of frog tadpoles and various fish. It was hoped to establish or eliminate these animals as the second intermediate host. One of the 8 tested was found infective for fish, and 1 infected tadpoles. These records do not elucidate the life cycles of these worms, but it is hoped that the work may help in the future study of strigeoid cercariae.

Descriptions of the cercariae used may be found in Brooks, F. G. (1948. Report to the Biology Division of the Wisconsin Conservation Department and the Department of Zoology, University of Wisconsin, on larval trematode parasites of Carrol Lake snails).

I wish to thank the Wisconsin State Conservation Department for assistance and the use of facilities at the Woodruff Fisheries Biology Laboratory.

Following is a list of the cercariae used and the results of the attempted infections:

1. *Cercaria lambda* Brooks, 1948:—Experimental metacercariae were not recovered from the following: Sunfish, *Lepomis gibbosus* (5), 1–48 days; bullhead, *Ameriurus* sp. (3), 15–38 days; perch, *Perca flavescens* (2) 1–3 days; Mud minnow, *Umbra limi* (2), 30–51 days; golden shiner, *Notemigonus chrysoleucas* (1), 30 days; frog tadpoles (sp?) (8), 6–20 days.

2. *Cercaria psi* Brooks, 1948:—Six sunfish and 2 largemouth black bass, *Huro salmoides,* developed metacercaiae identical or similar to *Neascus ambloplitis* = *Uvulifer ambloplitis* as recorded by Krull (1934, Copeia **2**: 69–71) and Hunter and Hamilton, (1941, Tr. Am. Micr. Soc. **60** (4) : 498–507).

Metacercariae were not recovered from the following—bullhead (1), 28 days; mud minnow (3), 34 days; perch (3), 3–11 days; stickleback, *Eucalia inconstans* (2), 7 days; golden shiner (4), 4–7 days; fathead minnow, *Pimephales p. promelas* (1), 5 days; frog tadpole (sp?) (4), 4–29 days.

3. *C. stonii* Brooks, 1943:—Experimental metacercariae were not recovered from the following—sunfish (4), 8–32 days; perch (1), 3 days; bullhead (3), 23–36 days; mud minnow (2), 10 days.

4. *C. douglasi* Cort, 1917:—Experimental metacercariae were not recovered from the following—sunfish (4), 3–30 days; fathead minnow (1), 20 days; golden shiner (1), 15 days.

5. *C. kappa* Brooks, 1948:—Experimental diplostomula were recovered from many frog tadpoles (sp?); they were recovered from the body cavity and were very similar to those reported for *Fibricola texensis* by Chandler (1942, Tr. Am. Micr. Soc. **61**: 156–167).

Experimental metacercariae were not recovered from the following—golden shiner (5), 4–6 days; sunfish (2), 7 days.

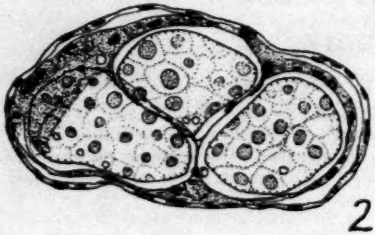
6. *C. macradena* Cort and Brackett, 1938:—Experimental metacercariae were not recovered from sunfish (2), 5–30 days.

7. *C. dohema* Cort and Brackett, 1937:—Experimental metacercariae were not recovered from the following—sunfish (4), 3–30 days; bullhead (1), 5 days; fathead minnow (2), 5 days; golden shiner (1), 20 days; mud minnow (1), 21 days.

8. *C. zeta* Brooks, 1948:—This form is very similar to *Cercaria Posthodiplostomum minimum* but experimental metacercariae were not recovered from the following—sunfish (6), 2–14 days; golden shiner (6), 2–12 days; mud minnow (4), 5–13 days; largemouth black bass (1), 12 days.—GLENN L. HOFFMAN, *University of North Dakota* [Present address: *Eastern Fish Disease Laboratory, Leetown (P. O. Kearneysville) West Virginia*].

PLATE I

1



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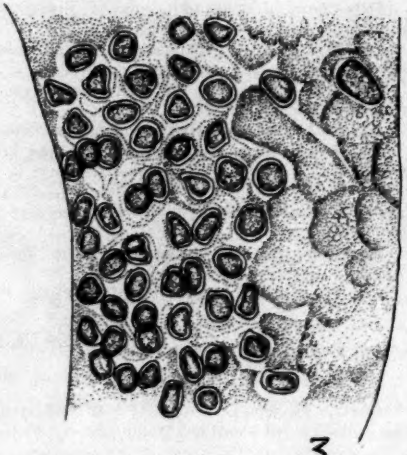

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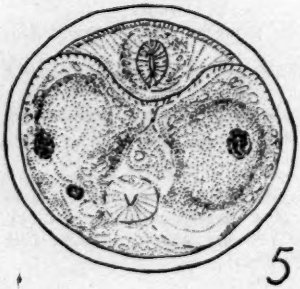

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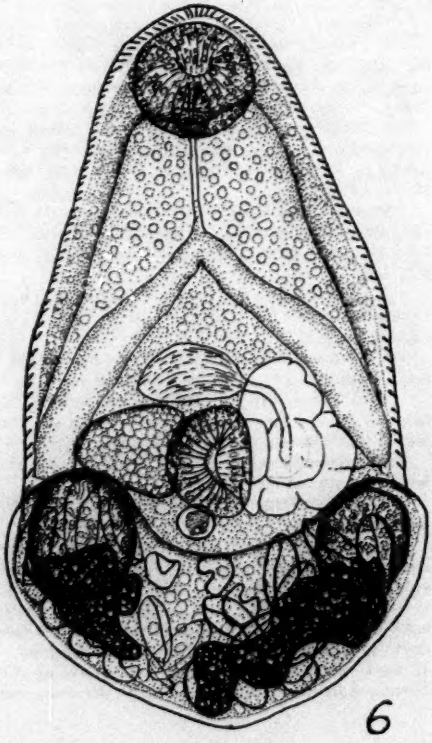

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THE INCIDENCE AND LOCATION OF METACERCARIAL CYSTS (TREMATODA: STRIGEIDA) ON 35 SPECIES OF CENTRAL NEW YORK FISHES

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Studies of the summer incidence and location of metacercarial cysts in marine and freshwater fishes have been made by Hoffman (1953 and 1956), Miller and McCoy (1930), and Wolfgang (1954). Fischthal (1949) observed tagged fish over a 6-month period (October to April) and noted the over-wintering of *Clinostomum* and *Neascus* spp. in *Perca, Ambloplites* and *Lepomis*. Since most collections in freezing latitudes are made during the warmer months of the year, more information on winter conditions seems desirable.

MATERIALS AND METHODS

All of the fish used in this study were caught wiwth a 10-foot seine in Catatonk Creek (Susquehanna River drainage) at Candor in Tioga County, New York, from November 11, 1949 to January 14, 1950. The collections were made in the same area of the stream and backwater, where the water depth varied from 1 to 3 feet. Water temperatures taken between 2 and 4 P.M. during this period ranged from 32° F to 50° F. Occasionally, it was necessary to break and remove the ice prior to seining. A total of 3,881 fish representing 7 families and 35 species were examined for metacercariae ("black spot") of strigeid trematodes. There were 1,696 fish collected in November, 1,538 in December, and 647 in January.

Parasite enumeration data were compiled by counting the darkly pigmented cysts visible on the left side of the body and fin regions of formalin-preserved fish.

Parasite determinations were not made from the 1949–1950 fish sample and subsequent attempts to identify the metacercariae from preserved fish proved fruitless. In April, 1957, additional fish were collected from the same area of Candor Creek and metacercariae were examined from the live fish. By placing excised cysts in a 0.5% acidified pepsin solution to digest the outer, thick-walled host cyst and then transferring the inner, resilient, hyaline cyst to trypsin solution, it was possible to obtain living metacercariae. Treatment with pepsin appeared to have little effect on the inner cyst. Identification of metacercariae obtained in this manner was attempted, using both living and stained preparations (Semichon's Carmine).

No attempt was made to identify all of the metacercariae recovered from the live fish but examination of 60 cysts from 3 species of fish (*N. cornutus, S. atromaculatus,* and *C. commersonni*) indicated that at least 2 and possibly 3 species of metacercariae were present. The most common metacercaria present was not readily referable to any of the species in Hoffman's key (1955), but closely resembled *Neascus nolfi* Hoffman. Until a more thorough study can be made of this worm it seems best to designate it as *Neascus* sp. A less common species was *Crassiphiala* (*Neascus*) *bulboglossa* (Van Haitsma). A third cyst unlike either of the preceding was found in 2 instances but attempts to recover the metacercariae from the cysts were unsuccessful. Since metacercariae were not determined for every fish species, it is possible that other larval trematodes of the black spot type were involved in our sample. For the Delaware and Susquehanna River drainages of New York, Hunninen (1936) reported *Neascus ambloplitis, N. vancleavei* and *Neascus* sp.

RESULTS AND DISCUSSION

A summary of the degree of infestation and location of metacercarial cysts on the fish is presented in Table I. Although the fish were taken from a relatively short section of stream there is much variation in the degree of infestation both

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TABLE I.—*Degree and location of metacercarial infestation*

Fish Host	Sample size	Length*	Weighted mean number of metacercaria on the left side of the fish						Range
			Head	Body	Fins				Total	Min.-Max.**
					Dorsal	Pectoral	Pelvic	Anal	Caudal		
Cyprinidae	3,073		1.79	18.43	1.40	1.05	.81	1.06	5.10	29.70	0–381
Semotilus a. atromaculatus	88	2–7	6.37	54.59	3.14	3.80	2.17	3.50	13.81	87.51	11–336
Nocomis micropogon	9	2–6	6.00	24.66	3.22	3.77	2.00	2.22	10.44	52.33	1–138
Rhinichthys a. atratulus	12	2–3	3.08	25.41	2.08	1.58	1.16	1.75	8.08	43.16	2–86
Leucosomus corporalis	651	2–6	2.28	29.97	1.10	1.33	.96	1.27	5.33	42.27	1–108
Notropis cornutus	964	2–6	2.34	18.01	2.69	1.34	1.32	1.68	7.63	35.15	0–381
Notropis analostanus	125	1–3	2.84	22.21	1.01	.39	.23	.49	6.31	33.50	0–133
N. cornutus × N.rubellus	270	2–5	.72	27.59	.71	.71	.50	.60	2.58	33.42	0–244
Exoglossum maxillingua	24	2–4	1.50	13.62	1.91	1.37	.79	1.25	4.25	24.70	1–106
Rhinichthys c. cataractae	12	2–3	.91	16.75	1.08	1.50	.50	.66	2.66	24.08	5–49
Campostoma anomalum	448	2–6	.69	4.20	.48	.72	.31	.36	3.05	9.83	0–93
Hyborhynchus notatus	43	1–3	.65	4.55	.41	.25	.18	.16	2.58	8.81	0–52
Notropis hudsonius	247	2–4	.71	5.87	.18	.23	.06	.10	1.35	8.59	0–150
Notemigonus crysoleucas	6	2–5	0	2.16	.16	.83	.50	0	1.33	5.00	0–10
Semotilus margarita	1	3	0	2.00	0	1.00	1.00	0	0	4.00	4
Notropis rubellus	123	2–4	.04	.96	.04	.03	0	.02	.03	1.14	0–12
Notropis amoenus	48	1–4	.02	.12	0	0	.02	0	0	.16	0–3
Clinostomus elongatus	1	6	0	0	0	0	0	0	0	0	0
Cyprinus carpio	1	6	0	0	0	0	0	0	0	0	0
Catostomidae	143		.90	6.39	2.78	1.05	.33	.41	5.73	17.62	0–109
Catostomus c. commersonni	127	2–9	1.01	7.12	2.53	.96	.28	.35	5.87	18.15	0–109
Hypentilium nigricans	14	3–9	0	.71	5.28	1.92	.78	.92	4.92	14.57	0–16
Erimyzon o. oblongus	2	4–6	.50	0	1.00	.50	0	.50	2.50	5.00	2–8
Percidae	51		.17	.84	.02	.09	.09	.04	.09	1.33	
Perca flavescens	4	4–5	.50	3.25	0	.50	.25	0	.50	4.50	2–8
Boleosoma nigrum olmstedi	46	2–3	.15	.63	.02	.06	.08	.04	.06	1.06	0–12
Hadropterus p. peltatus	1	6	0	1.00	0	0	0	0	0	1.00	1
Centrarchidae	568		.32	1.43	.17	.15	.06	.09	.56	2.82	
Lepomis gibbosus	305	1–6	.45	2.11	.22	.21	.10	.13	.81	4.07	0–27
Micropterus salmoides	91	2–8	.37	1.56	.27	.21	.06	.10	.67	3.23	0–26
Pomoxis nigro-maculatus	1	4–5	0	1.00	0	0	0	0	0	1.00	1
Micropterus dolomieu	27	2–7	.03	.14	.11	.03	.03	.03	.25	.66	0–8
Ambloplites rupestris	124	1–4	.07	.14	.02	.01	0	.01	.04	.34	0–5
Lepomis auritus	20	1–3	.05	.10	0	.05	0	0	.05	.25	0–3
Ameiuridae	9		0	.22	0	0	0	0	0	.22	
Shilbeodes marginatus	7	2–5	0	.28	0	0	0	0	0	.28	0–1
Amelurus nebulosus	2	3–4	0	0	0	0	0	0	0	0	0
Esocidae	12		0	0	.08	0	0	0	.16	.24	
Esox niger	12	7–12	0	0	.08	0	0	0	.16	.24	0–3
Cottidae	25		0	0	0	0	0	0	0	0	
Cottus cognatus	23	1–4	0	0	0	0	0	0	0	0	0
Cottus bairdi	2	2–3	0	0	0	0	0	0	0	0	0
Total—All Species	3,881		1.50	15.05	1.24	.90	.66	.87	4.33	24.60	0–381

* Range in total length in inches.
** Range in number of metacercarial cysts on the left side of the fish.

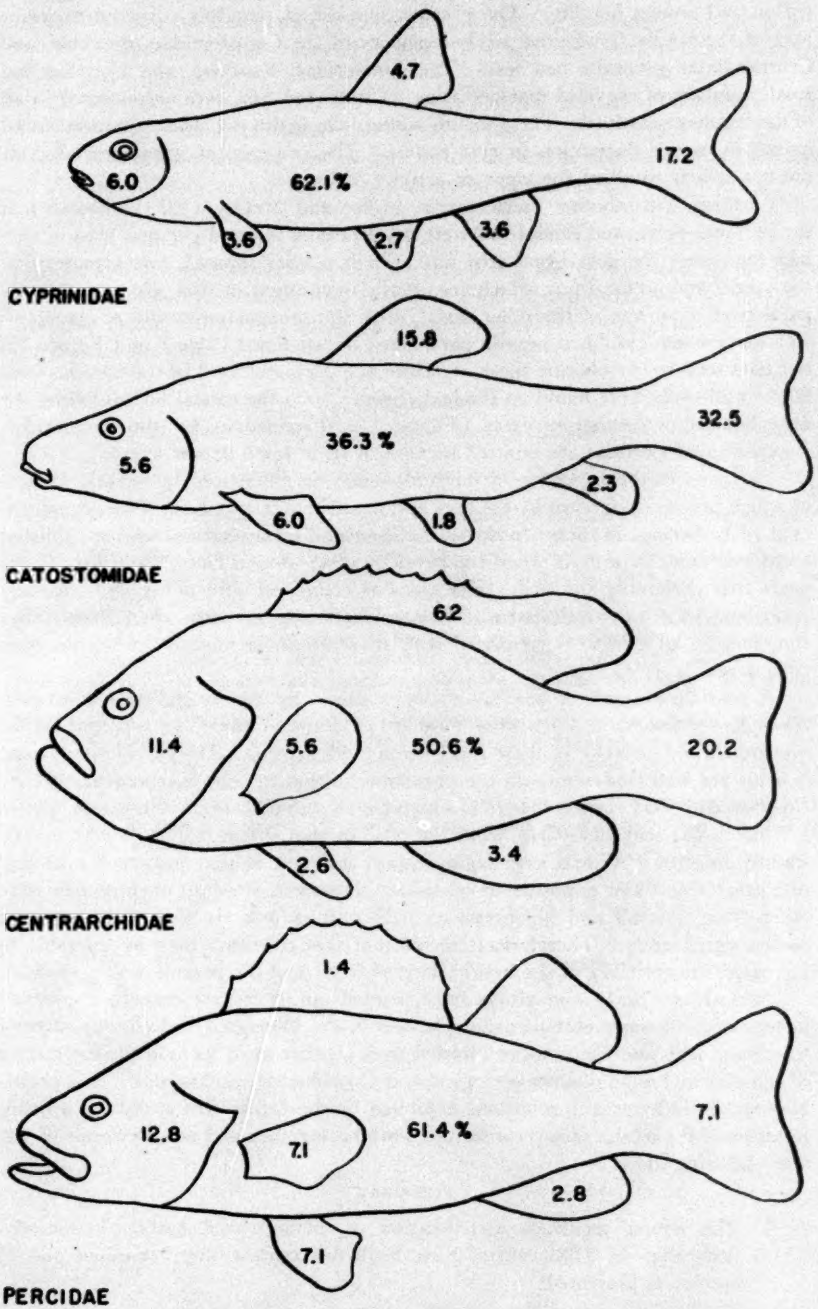

FIGURE 1. Percentage distribution of metacercarial cysts on the left side of the fish.

within and among families. The greatest number of parasites occurred in members of the family Cyprinidae while members of the Catostomidae, Percidae, and Centrarchidae generally had less. The Ameiuridae, Esocidae, and Cottidae had small numbers of encysted metacercariae. Uninfested fish were encountered in all of the families examined. The 3-month winter sample did not show any pronounced month to month fluctuation in cyst counts. The presence of metacercarial cysts did not appear to affect the vigor or activity of the fish.

Working with another metacercaria, Miller and McCoy (1930) showed that the pectoral, pelvic and caudal fins were more heavily infested per unit area of surface than were the dorsal and anal fins. Their studies showed, furthermore, that the caudal and pectoral fins, which are usually in constant motion, are more heavily parasitized than any of the other fins. With the possible exception of the Catostomidae, which exhibited heavily parasitized dorsal fins (Table I and Figure 1), our data tend to corroborate those of Miller and McCoy. In 3 of the families over 50% of all cysts were found on the body proper, with the caudal fin exhibiting the next highest percentage of cysts (Figure 1). Percentages for the Ameiuridae, Esocidae, and Cottidae are omitted because of their small values.

Variation in the incidence of infestation may be influenced by several factors, of which habitat preference by the host and possible host specificity must be considered. The bottom dwelling *Ameiurus*, *Shilbeodes*, *Cottus*, and *Boleosoma* exhibited a reduced number of cysts when compared to other stream fish (Table I). It appears that preference for swift, riffle areas as compared with non-riffle areas may contribute to a lower infestation. This relation may be seen when *Rhinichthys cataractae*, a riffle form is compared with *R. atratulus*, a minnow of slower, non-riffle areas.

A possible example of host specificity is shown by the natural hybrid between *Notropis cornutus* and *N. rubellus* (Table I). Raney (1940) has reported on the occurrence and behavior of these species and their hybrids. The parent species and hybrids are well represented in the present collection by 964 *N. cornutus*, 123 *N. rubellus*, and 270 hybrids. There is a high degree of infestation of 1 parent species (*N. cornutus*) and the hybrid, while the other parent species (*N. rubellus*) is only slightly infested. At first one might suspect different habitat preferences leading to a greater or lesser exposure to cercariae. However, since all of these fish were taken from a small and homogeneous habitat it is unlikely that their exposure varied significantly. Therefore, it seems that this condition may be referable to increased susceptibility of the hybrid derived from 1 of the parents (*N. cornutus*).

As LaRue (1951) and others have pointed out, there are numerous complex factors that influence host-parasite relations in the Digenea. Indeed, any attempt to explain why one fish is more infested than another must include the interaction of complex and often obscure ecological and physiological mechanisms. It is probable that the differential infestations exhibited by the fish in this study are a manifestation of the effects of several factors working together and not the result of any one operating alone.

SUMMARY

1. The winter incidence and location of metacercarial cysts (Trematoda: Strigeida) on 3,881 central New York fish representing 7 families and 35 species is presented.

2. The fish families, arranged in order of decreasing numbers of parasites, are: Cyprinidae, Catostomidae, Percidae, Centrarchidae, Ameiuridae, Esocidae, and Cottidae.
3. The body proper exhibited the highest number of cysts with the caudal fin having more cysts than any of the other fins.
4. A brief discussion of host-parasite relations, and a possible case of increased susceptibility as a result of hybridization, is presented.

ACKNOWLEDGMENTS

The authors wish to acknowledge the taxonomic advice of Glenn L. Hoffman, University of North Dakota, who examined some of our preparations; Royal D. Suttkus, Tulane University, for his help with the field collecting; and Erica Evans for making the cyst counts.

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PROECHINOCEPHALUS TARAI N. GEN., N. SP. A NEW TREMATODE (ECHINOSTOMATIDAE) FROM AN INDIAN EGRET, *BULBULCUS IBIS COROMANDUS*

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A single specimen of the trematode described below was obtained from the intestine of an Indian cattle egret, *Bulbulcus ibis coromandus,* which was examined in January, 1950. This study is thus based only on the whole mount of the worm. The infection with this species is rare, as out of about 150 cattle egrets examined from the suburbs of Allahabad during different seasons, only 1 host yielded a single specimen of the parasite, the prevalence of infection being 0.6%.

Because of its resemblance to the genus *Chaunocephalus,* in the symmetrical disposition of the testes, the parasite is referred to the new genus *Proechinocephalus.* The species is described as *Proechinocephalus tarai* after the name of the village Tarwa, where the host was obtained.

Proechinocephalus tarai n. gen., n. sp.
(Figs. 1 and 2)

Description (Based on one complete specimen; measurements are in mms.)

Body elongate 7.42 by 2.09; more or less flattened with extremities rounded and greatest breadth almost uniform in middle third of body. Cephalic collar terminal; 0.37 by 0.6, small but better set off in living unpressed specimen. Collar spines 23, arranged alternately in double rows with no dorsal interruption. Of these, the 4 corner spines on each side are larger than lateral and dorsal spines, which are short; largest collar spine measures .095 × .027. Spines lack straight margins and have bluntly pointed ends. Body spines present all over body and usually smaller than collar spines. Oral sucker subterminal, small, rounded and 0.27 in diameter. Acetabulum pre-equatorial and located 2.08 from anterior end and 0.56 behind intestinal bifurcation; it is strongly muscular, spherical and protrusible measuring 0.96 in diameter; size ratio of the 2 suckers 1 : 4.

A funnel-like prepharynx 0.05 × .013 present. Pharynx longer than broad, measuring 0.255 × 0.204. Esophagus 1.96 long and divided into an inverted flask-shaped anterior part with maximum breadth 0.32 and a posterior slightly curved neck-like tubular part 0.96 broad at its posterior termination. Intestinal bifurcation, situated 1.52 behind anterior end, separated from acetabulum by intervening genital opening. Intestinal ceca only slightly sinuous and extend to posterior end of body with their width gradually increasing posteriorly. Ceca wide apart in pretesticular region, closer together in the posttesticular region, partly due to narrower breadth of body in that region and partly due to their inner margins becoming slightly bulged.

Excretory vesicle is Y-shaped, median stem reaching anteriorly to testes before bifurcating into forks. Excretory pore terminal. Genital pore conspicuous, ventral and located more to right side than median. Situated 1.71 from the anterior end, it lies halfway between the acetabulum and intestinal bifurcation. Testes symmetrical, intercecal, and more or less globular with smooth and entire margins, situated at the posterior boundary of the middle third part of body 4.875 in front of hind end. The 2 testes, 0.45 in diameter, almost equal, postovarian, and separated from each other by 0.15 distance which is traversed by a few obliquely running muscle fibers. A small cirrus sac 0.375 by 0.225 situated in front of acetabulum extends diagonally from its anterolateral margin to genital pore. Cirrus eversible; everted cirrus beset in its proximal part with spines measuring 0.018–0.025, and bulged distally. Ovary submedian, pretestcular and intercecal and lies close to right cecum at a distance of 0.6 behind acetabulum and 0.45 in front of right testis. It is slightly transversely oval, smaller than testis and measures 0.25 × 0.3. Oviduct arising from posterior end of ovary runs towards the median line for a short distance to joint oötype surrounded by large Mehlis' gland mass. Laurer's canal

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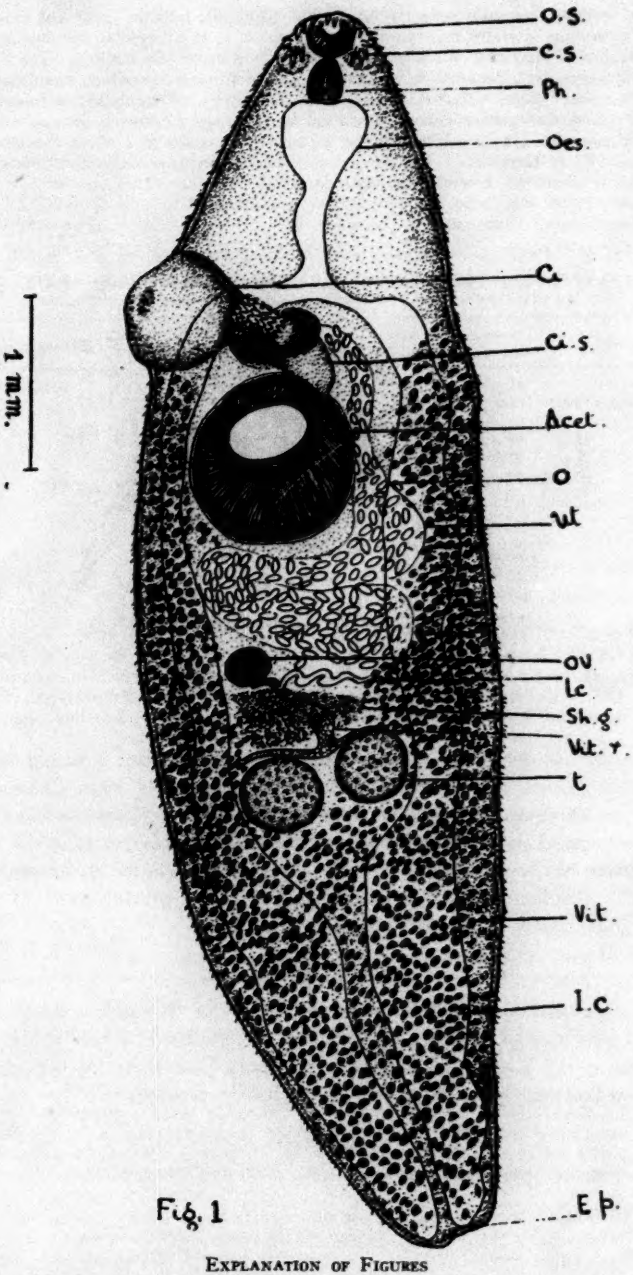

EXPLANATION OF FIGURES

(Both the figures were made with the aid of camera lucida.)

FIGURE 1. *Proechinocephalus tarai*, whole mount.

present and seminal receptacle seems to be lacking. Vitelline follicles small and close together, extensive, extending laterally in pretesticular region to level of genital opening and in post-testicular region to hind end, filling entire space by meeting in the midline. The 2 transverse yolk ducts situated just anterior to testes meet to form yolk reservoir which has form of a tubular sac with a short anterior duct connecting with the oötype. Thin-walled uterus arising from the Mehlis' gland mass makes a few intercaecal loops in space between genital field and acetabulum. Uterus, filled with a large number of eggs, terminates in a short slender metraterm which lies dorsal to cirrus sac. Eggs ovoid, yellowish, and measure 0.05–.09 × 0.06–0.1.

Host: Bulbulcus ibis coromandus, cattle egret.

Location: Small intestine.

Locality: Tarwa, Allahabad.

Discussion. *Proechinocephalus* is clearly a member of the family Echinostomatidae and is most closely related to *Chaunocephalus* Dietz, 1909 on account

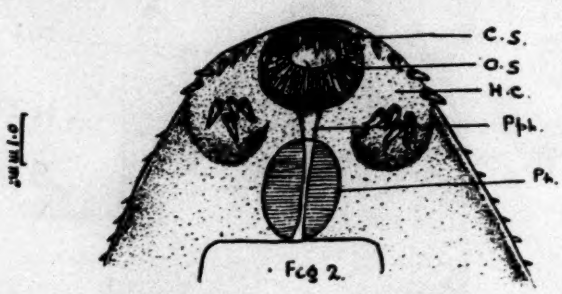

FIGURE 2. *P. tarai,* enlarged view of the anterior portion of the body.

Abbreviations: Acet.—Acetabulum; Ci.—Cirrus; Ci.S.—Cirrus sac; C.S.—Collar spine; E.P.—Excretory pore; H.C.—Cephalic collar; I.C.—Intestinal cecum; Lc.—Laurer's canal; O.—Eggs; O.S.—Oral sucker; Ov.—Ovary; Ph.—Pharynx; Pph.—Prepharynx; Sh.g.—Mehlis' gland mass; t.—Testis; Ut.—Uterus; Vit.—Vitellaria; Vit.r.—Vitelline reservoir.

of the symmetrical disposition of the testes in which character it stands apart from all the other genera of the family. It differs remarkably from *Chaunocephalus* in having an elongate body not divisible into an anterior large swollen part and posterior elongated part. It is further separated from this genus in the more anterior position of the acetabulum and the absence of vitellaria in the preacetabular region, in the number of collar spines being 4 less, and greater width of the intestinal ceca particularly in the post-testicular region.

ACKNOWLEDGMENTS

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RESEARCH NOTES

NOTES ON AVIAN HIPPOBOSCIDS

An opportunity to observe avian louse-flies was afforded in August, 1948 and 1950, in Algonquin Park, Ontario, when Dr. W. W. H. Gunn live-trapped and banded a number of birds, mainly sparrows. *Ornithomyia fringillina* Curtis* was found on juvenile and adult sparrows of 3 species, *i.e.*, eastern chipping, *Spizella p. passerina* (Bechstein); white-throated, *Zonotrichia albicollis* (Gmelin) and song *Melospiza m. melodia* (Wilson) but *Ornithoica vicina* (Walker) was only on song sparrows (Table I). Other records of hippoboscids on birds are mentioned in Table I, that of *Ornithoctona fusciventris* (Wiedemann) on Scarlet Tanager (*Piranga erythromelas* Vieillot) being a new Canadian host record. Two specimens of the flies were taken from man on days when no birds were being handled. These flies were *Or-*

TABLE 1—*Species of hippoboscids collected from birds in Algonquin Park (unless otherwise indicated) including date of collection, longevity, species of bird and number of flies per bird*

Species of Hippoboscid	No. of Flies	Species of Bird	No. of Birds	Date of Collection	Remarks
Ornithomyia fringillina	2	Song Sparrow	2	12/8/48	
	1	" "	1	17/8/48	died 24/8/48
	8	Song Sparrow (No. 1 & No. 2)	2	20/8/50	
	4	" " (No. 3)	1	20/8/50	
	1	" "	1	20/8/50	
	3	" " (No. 2)	1	21/8/50	
	3	White-throated Sparrow	1	2/8/50	one died 5/8, two on 7/8
	3	" " "	1	2/8/50	" " 7/8, one on 8/8 one on 10/8
	0	" " "	1	5/8/50 }	same bird recaptured
	0	" " "	1	6/8/50 }	
	1	" " "	1	5/10/50	Toronto, died 12/10/50
	2	Chipping Sparrow	1	20/8/50	
	2	" "	1	20/8/50	
	2	" "	1	20/8/50	
	3	" "	1	20/8/50	
	0	" "	1	20/8/50	
Ornithoica vicina	1	Song Sparrow	1	10/8/48	
	4	" "	2	12/8/48	
	4	Song Sparrow (No. 1 & No. 2)	2	20/8/50	
	1	" " (No. 3)	1	20/8/50	
	1	Bronzed Grackle	1	20/8/50	
	0	" "	1	20/8/50	
	1	Barred Owl	1	11/10/33	Arden, Ontario
Ornithoctona fusciventris	1	Scarlet Tanager	1	14/6/44	Toronto region (coll. by C. E. Hope)
Lynchia americana	3	Long-eared Owl	1	4/11/44	Toronto region (coll. by C. E. Hope)
	1	Ruffed Grouse	1	13/10/51	Cornwall, Ontario

nithomyia fringillina (17/8/49) and *Ornithoica vicina* (25/8/48). The position of the flies on the avian host was not seen because they flew from the birds faster than the eye could follow when the birds were handled. They flew often to windows or to the underside of shelves.

One song sparrow from which several *O. fringillina* were removed, had 3 additional ones the following day. This may indicate that these flies move readily from host to host, a factor important in the transfer of blood parasites, or ectoparasites such as Mallophaga. One *O. fringillina* collected from a song sparrow on August 20, 1950, carried a male biting louse (Bequaert, 1953, Entom. Am. **32**: 1–209) dorsally on the right side near the base of the abdomen. It was identified** as *Brüelia* sp. Bequaert (1953) refers to other cases of phoresy involving *Brüelia, O. fringellina* and the song sparrow.

Individuals of *O. fringillina* collected in August 1949 and 1950 would not feed on the defeathered backs of week-old Pekin ducks on the day of collection nor the day after. However, some success was obtained in keeping flies alive in "longevity" tubes (Davies, 1953, Canad.

* Dr. J. C. Bequaert kindly identified or confirmed identification of the hippoboscids.

** Professor E. W. Stafford, Mississippi State College kindly identified the louse.

J. Zool. 31: 307) in the laboratory. A specimen of *O. fringillina* was kept alive at room temperature for 6½ days in 1948 and one for 4½ days in 1949. Flies collected on August 2 and 5, 1950, were kept alive at room temperature for 2 to 5 days. One female collected on October 5, 1950, from a dead white-throated sparrow at Toronto, Ontario, lived for 7 days in a longevity tube at 50° F. These flies were not seen to feed on the sucrose crystals provided nor on the water-soaked wick.

The female of *O. fringillina* collected from a dead white-throated sparrow on the morning of October 5, 1950, was kept under observation at the Ontario Research Foundation, Toronto. At 9 a.m. the next day the female had a dark puparium well-protruded from the abdomen. As the fly was active with a strong positive phototaxis, a dark sleeve was placed around the "longevity" tube to reduce the attraction to the top of the tube that, it was thought, might have interfered with the deposition of the puparium. At 3 p.m. the puparium was on the bottom cotton-plug of the tube. The adult, which was still moving actively at the top, was transferred to another tube. The puparium was kept in the tube at an average temperature of 70° F. Four months later, between February 6–12, the adult emerged. Cornelius (1869) in Bequaert (1953) reported an even longer pupal period for *Ornithomyia biloba* in swallow nests in Germany, from fall until the next March or even May. Also Shipley (1913) in Bequaert (1953) surmised that in England *O. fringillina* puparia overwinter in red grouse nests, the adults emerging in June after almost 9 months. The puparia laid in the summer, however, may produce adults in a much shorter time as is found in other species.

Bennett (personal communication, 1956) in carefully examining about 2500 nests of a variety of bird species for *Protocalliphora* in Algonquin Park has yet to find puparia of hippoboscids. Actually the *Protocalliphora* study developed from an initial investigation by the author for hippoboscid puparia in birds' nests, including many swallows' nests, but no hippoboscid puparia were found. This apears to be the general condition in North America especially in regard to *O. fringillina* (Bequaert, 1953). On the other hand Bequaert (1953) presents a number of references to *O. fringillina, O. biloba* and other avian hippoboscids in Europe regularly larvipositing in birds' nests.—DOUGLAS M. DAVIES, *Department of Biology, McMaster University, Hamilton, Canada.*

OBSERVATIONS ON THE INFECTION OF THE SHRIMP, *PENAEUS SEMISULCATUS*, BY *EPIPENAEON ELEGANS* IN THE PERSIAN GULF

In the course of fishery investigations in the western Persian Gulf during September and October, 1956, the shrimp *Penaeus semisulcatus* de Haan was observed to be parasitized by a large bopyrid isopod. This was subsequently identified as *Epipenaeon elegans* Chopra, a form previously described only from *Penaeus carinatus* Dana (*P. monodon* Fabricius) and *Penaeus semisulcatus* in the Bengal estuary and Ganges delta of India. The material reported here was collected at Abu Ali and Tarut Bay, Saudi Arabia.

The female bopyrids ranged in length from 6 mm to 20 mm and were found in the branchial cavity, between the branchiae and exoskeleton, of the host. In every case, infection was by a large female and an associated diminutive, 2 mm to 7 mm, male. Parasitized shrimp could readily be detected by an obvious deformation of either the right or left side of the cephalothorax.

Although *Penaeus japonicus* Bate, *Penaeopsis* (*Metapenaeopsis*) *novaeguineae* (Haswell) and an unidentified species of *Metapenaeus* were also taken in significant numbers, bopyrid parasites were found only in *Penaeus semisulcatus.*

One hundred and twenty-one infected shrimp, representing a total parasitism of 2.5%, were found in a sample of 4900 *P. semisulcatus.* A tendency toward more frequent infection with increased total length was apparent. If the parasitized individuals in each size class are taken as a percentage of the total number for that class in the sample population (Fig. 1). percentage infection varied with host size from 0.3% at 93 mm to 100% at 163 mm. Comparison of the total length frequencies of the sample population and of the parasitized shrimp (Fig. 1) also shows that infection was most common in larger shrimp. Although the size range of the sample was from 33 mm to 163 mm, no parasitism was observed in shrimp under a total length of 93 mm. Parasitized shrimp were almost equally divided between males and females, and no significant differences were found in the length frequencies of infected males and females.

The effects of *Epipenaeon* infestation on the *Penaeus semisulcatus* population are unknown, but this isopod could conceivedly cause a high mortality among adult shrimp. In the absence of further data it can only be stated that infestation by *Epipenaeon elegans* probably reduces the survival potential of the host, and that there is, at present, no means of ascertaining whether the observed 2.5% infection represents a maximum or only those hardy individuals surviving parasitism.

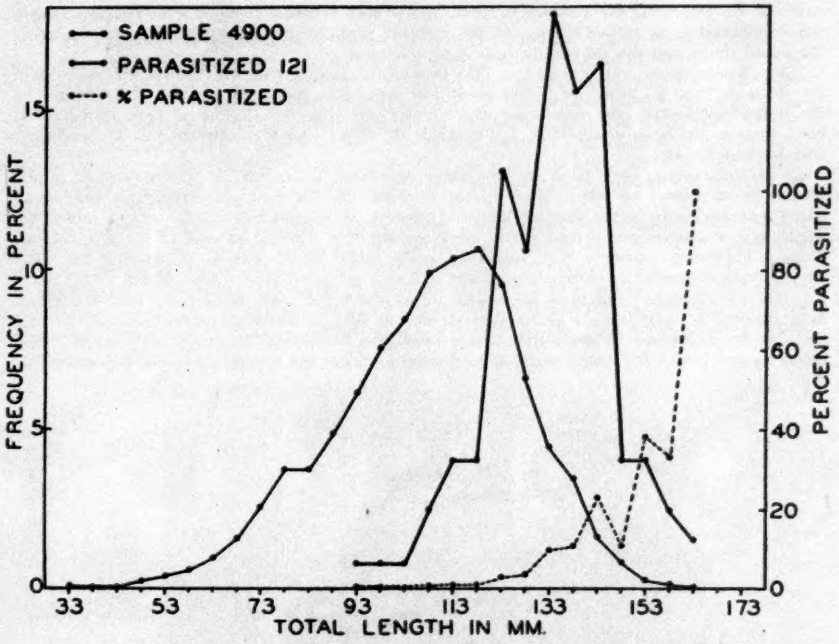

FIGURE 1. Total length frequencies of parasitized and sampled population of *Penaeus semisulcatus* together with percentage of shrimp parasitized by size class. Figures indicate numbers of individuals.

My thanks are extended to Dr. David Causey and Dr. Sueo M. Shiino for identification of the bopyrid and to Dr. Fenner A. Chace Jr. for his identification of the host.—C. E. DAWSON, *Bears Bluff Laboratories, Wadmalaw Island, S. C.*

THE CULTIVATION OF *TRYPANOSOMA CONGOLENSE* IN VITRO

Media previously used for the cultivation of *Trypanosoma congolense* were variations of a liquid medium prepared by combining citrated blood with Ringer's solution (Ponselle, 1924, Compt. rend. Acad. Sci., Paris **178:** 1219–1221; Reichenow, 1934, Arch. Schiffs-u. Tropen-Hyg. **38:** 292–302; Brutsaert and Henrard, 1936, Ann. Soc. Belge. Méd. Trop. **16:** 457–478). The forms growing in these cultures were studied by Reichenow (1934, Arch. Schiffs-u. Tropen-Hyg. **38:** 292–302) who described them as similar to those found in the gut of *Glossina*. In the fly these stages are not infective. The capacity of culture forms to develop in *Glossina* was also studied by Reichenow (1939, Arch. Schiffs-u. Tropen-Hyg. **43:** 197–202) who concluded that they were capable of undergoing initial development in the tsetse fly and only rarely capable of completing development. Henrard and Peel (1950, Bur. Perm. Interafr. Tsétsé et Trypan. 122/T) reported that though African trypanosomes lose their pathogenicity in culture within 24 hours, infectivity can be restored by feeding clean flies on the culture material, but that this must be done during the first transfers since successful transmission to the fly decreases due to ageing *in vitro*. However, Brutsaert and Henrard (1936) stated that they infected 1 out of 25 tsetse flies, and 2 goats from a strain which had been maintained *in vitro* for 156 days.

Physiological and immunological studies on *T. congolense* have been confined to the adult trypanosome from the blood stream. The lack of similar studies on culture forms is not due to lack of interest in this species but undoubtedly to the fact that the medium previously used did not lend itself to this purpose since the flagellates congregate at the surface of the settled red cells and can not be readily separated from the red cells in the medium.

In order to establish *T. congolense in vitro*, attempts were made to grow it in a liquid medium similar to those previously used, which with this strain did not prove successful, and in diphasic blood agar media. In this note we are reporting successful cultivation of the Lumu

strain of *T. congolense* for a period of more than a year in diphasic blood agar medium, which can be adapted to *in vitro* studies. In the diphasic medium the flagellates grow suspended in the liquid phase and can be readily harvested and used.

The Lumu strain was provided in 1955 through the courtesy of Dr. Robert Desowitz from the West African Institute for Trypanosomiasis Research, Northern Nigeria. At the time of the isolation reported here the strain was 5 years old. The diphasic blood agar medium has been reported by Tobie *et al* (1950, J. Parasitol. **36**: 48–54) for the cultivation of *T. gambiense* and *T. rhodesiense*.

For isolation *in vitro* from its laboratory host (the white rat) it was necessary to use human blood instead of rabbit blood in the medium. In the first successful isolation human blood was used only in the original tubes. However, when attempts were made to repeat the isolation it was necessary to use human blood for the first transfer as well as the original isolation. Following transfers then succeeded when rabbit blood was used although for some time growth continued to be more luxuriant in media prepared with human blood.

Not all attempted isolations succeeded. those which did were made from rats which had been infected at least 3 days and which showed at least a moderate parasitemia of actively dividing trypanosomes. When isolation was attempted in medium prepared with rabbit blood, parasites survived a few days; dividing forms were evident but subcultures were not successful.

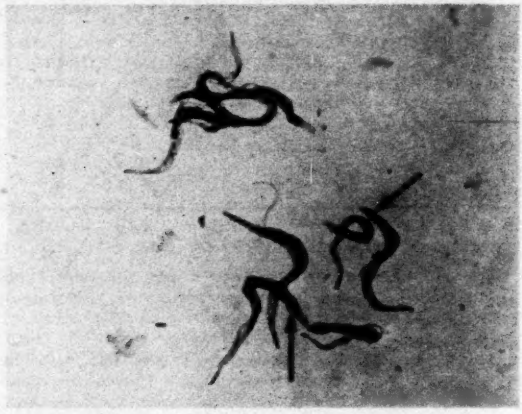

Figure 1. Noninfective slender trypanosomes of *T. congolense* from culture. Notice kinetoplast posterior to nucleus: Giemsa stain, × 1815

First subcultures were not successful when made 7 days after isolation but were any time after 10 days as long as parasites appeared in good condition. Transfers are now made regularly between 8 and 12 days. Cultures are incubated at a constant temperature of 24°–25°C.

Studies on the morphology of the *in vitro* forms confirm Reichenow's description (Figure 1). The usual stain used for trypanosomes is Giemsa which also stains the numerous volutin granules in many of the culture forms. This frequently made identification of the kinetoplast difficult. In order to eliminate this staining of the granules and thus definitely distinguish the kinetoplast, Feulgen's stain (Lillie, 1954, The Blakiston Company, Inc., New York.) was also used.

Those forms in which the kinetoplast was visible by Feulgen's staining were obviously trypanosomes. The length of the body as well as the flagellum, when it was free varied somewhat and occasionally more stubby forms were seen. Since these cultures were not infective to rats, these forms probably correspond to the stubby forms found in the midgut of the fly.

Since there is some discrepancy in earlier reports on the infectivity of *T. congolense* culture forms, tests of the infectivity of these cultures were made. White rats inoculated with material from 1- to 4-day old isolation cultures remained negative as did rats inoculated with material from transfer cultures after the strain had been maintained *in vitro* for 87 days and for 294 to 306 days. It is obvious that adult trypanosomes of *T. congolense* rapidly change form and become noninfective when isolated *in vitro*.—ELEANOR JOHNSON TOBIE (with the technical assistance of HARVEY D. JAMES JR.), *Laboratory of Tropical Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda 14, Maryland.*

A NEW METHOD FOR EXAMINING SNAILS FOR TREMATODE PARASITES

The examination of snails for their trematode parasites has consisted of either crushing the shell of the snail and examining the digestive gland and gonads or isolating the snail in a dish of water and later examining the water for cercariae. The former method sacrifices the snail and destroys a source of parasitic material which may be exceedingly valuable. This method also may reveal seemingly mature cercariae which are in reality not ready to be shed (Stunkard 1930, Parasitology **22**: 268–274). The latter method is quite time-consuming and requires large quantities of glassware even when several snails are placed in 1 dish as suggested by Rees (1931, Parasitology, **23**: 428–440); it also overlooks those trematode infections which are not at a stage for shedding cercariae.

During the examination of large numbers of the stream snail, *Oxytrema silicula* (Gould), a technique of examination was developed. *O. silicula* has a sturdy shell and a tall spire. In nature the apexes of nearly all are eroded but have become closed.

The method is as follows: The tip of the shell is ground away on an electrically driven carborundum wheel, such as is found in a work-shop, until a small part of the digestive gland is removed (see line AA, Fig. 1). Heat from the friction of the high-speed grinding wheel

Fig. 1

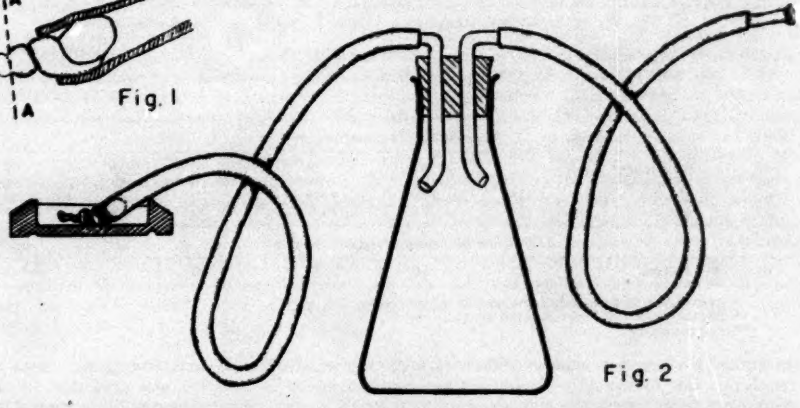
Fig. 2

cauterizes the injured tissues. The snail is then placed in a dish of water to await examination. The aperture end of the snail is then placed in the end of a rubber tube in such a way that the ground tip is outside. This tube is connected through a 2-holed stopper to a bottle which serves as a trap to prevent water from entering the mouth of the operator. (See Fig. 2) A tube leads from the other hole in the stopper to the mouth of the operator. The snail in the end of the tube is then immersed in a small dish of water on the stage of a dissecting microscope and gentle pressure is exerted on the aperture of the snail through the tube. This pressure forces the hepato-pancreas of the snail out of the shell into the water for examination. Shell fragments from the grinding may impair the free movement of the snail tissues but they can be removed easily with fine forceps or a dissecting needle. Rediae or sporocysts can be seen on the surface of the hepato-pancreas and can be removed with fine forceps. In some cases the parasites can be identified without removing them from the snail. In light infections the rediae and sporocysts seem to be more plentiful along the inner curvature of the gland in the vicinity of the ducts that lead anteriorly from the gland. While examining the snail for parasites, care must be taken not to tear the soft tissues of the snail. When one has finished examining the tissues, gentle suction on the tube will draw the hepato-pancreas back into the shell. The snail can be dried in the air a few minutes and the hole sealed with a little paraffin or tacky wax.

With this technique as many as 300 snails have been examined in 1 afternoon. Both early and late infections can be diagnosed and the snail need not be sacrificed.

A check for mortality revealed that the snails were weakened by the operation but that they lived long enough to permit a great deal of life-history work to be completed. To test for mortality 164 snails were examined by this technique and then kept in aerated aquaria at 16° C; i.e. under conditions at which snails are usually kept in this laboratory. At the end of 14 days 155 snails were still alive; at 29 days 103 were alive; at 36 days 69 were alive and at 58 days

49 were alive. Death of the snails appeared to be due to a *Saprolegnia* infection, this presumably entering the snail through injured tissues. There appeared to be little difference in mortality rate between those "stoppered" with tacky wax and melted paraffin.

This technique may overlook infection in tissues other than the hepato-pancreas, but it will detect many infections that the isolation technique will overlook. Measures to control the fungus in the aquaria should markedly increase the useful life of the snails. (This work has been supported by PHS grant E-867.)—JAMES E. MCCAULEY, *Department of Zoology, Oregon State College, Corvallis, Oregon.*

IMMUNITY IN CATTLE TO THE LUNGWORM, *DICTYOCAULUS VIVIPARUS*: A TEST OF THE PERSISTENCE OF ACQUIRED RESISTANCE

In an earlier communication, Rubin and Weber (1955, Proc. Helminth. Soc. Wash. **22**: 124–129) reported that the intravenous administration of 5.0 ml/lb body weight of immune serum enabled 2 calves to survive a known lethal dose of 50,000 lungworm larvae. Two years after elimination of the lungworms acquired from this dose of larvae, each of these animals (Nos. 42 and 44, Table 1), then 30 months old, was reexposed to a similar number of larvae in

TABLE 1—*Results of exposure of cattle to lungworms*

Animal No.	Type of exposure	Weight changes		Prepatent period (days)	Worms recovered			
		2 weeks (lb)	1 month (lb)		Number	Length in mm. 0–2 %	2–15 %	15–50 %
50,000 larvae administered								
42	Challenge*	+ 7	+ 37	**	35	100	0	0
44	Challenge*	+ 8	+ 17	**	19	89	11	0
12	Initial	−118	−305	23***	9,470	2	44	54
21	Initial	+ 25	+ 31	26	55	49	25	26
100,000 larvae administered								
65	Initial	−117	− 56	**	0	..	..	..

* 24 months after elimination of initial infection.
** The infection did not become patent.
*** Approximate.

an effort to determine whether either or both of them had acquired a lasting resistance to reinfection due to this prior infection; accidental exposure to infection was precluded by the conditions under which the animals were kept at all times. Simultaneously, as a control on the factor of age, 2 other cattle (Nos. 12 and 21) that were also 30 months old, but had never been exposed to infection or received immune serum, were given the same number of larvae. An 8-year-old cow, No. 65, with a history that, so far as was known to the writer, precluded prior exposure to lungworm infection, was given 100,000 larvae. The animals were autopsied 1 month after the larvae were administered. The data obtained bear not only on the main point at issue, but also on the phenomenon of inhibition of the development of the parasite as an expression of resistance and on the role of the host's age in resistance to lungworm infection and disease, a point on which reports conflict. (For bibliography, see: Rubin and Lucker, 1956. Am. J. Vet. Res. **17**: 217–226)

Neither of the reexposed animals showed pronounced clinical symptoms or ante-mortem parasitological evidence of infection. Only a few worms, all small and immature, were recovered from each of them, and none of the lung lesions present were recent. Each of the 2 initially exposed 30-month-old animals acquired a patent infection, but these animals differed markedly from one another in their responses to infection. The weight gain of No. 21 was normal during the first 2 weeks; during the remaining 16 days its rate of gain declined. Coincident with this decline were a continuously elevated temperature, persistent coughing, and increased respiration. Thus the clinical effects increased in severity as the interval after infection increased. Although this animal did not harbor at autopsy significantly more worms than the animals reexposed to infection, the fact that it acquired a patent infection, the clinical effects mentioned, the relative degree of lung involvment, and the greater size of the worms recovered, all indicated its lesser degree of resistance to infection.

Animal No. 12 was severely affected with lungworm disease and was slaughtered *in extremis*. In the last 2 weeks preceding autopsy, it refused all food and lost almost 200 pounds in weight. A very large number of worms, many of those in the bronchi being large specimens, were recovered from its lungs, which were almost completely pneumonic.

Cow No. 65 did not acquire a patent infection and was negative for lungworms at autopsy; no recent lesions were observed in the lungs. Some larvae probably reached its lungs, however, since breathing became pronouncedly labored about 1 week after infection. This, along with anorexia, weight loss, and an elevated temperature, continued until the end of the 2nd week, at which time the apparent infection was eliminated. Thereafter, the animal gained weight rapidly and became completely asymptomatic.

In the interim between their first and challenge exposures to infection, the resistance of animals Nos. 42 and 44 had undoubtedly increased, since both acquired patent infections originally (Rubin and Weber, 1955). Whether their strong resistance when 30 months old was due primarily to age or was primarily a result of the prior administration of larvae is not clear in view of the behavior of the 2 age controls. The experiment does demonstrate definitely, however, that attainment of this age does not by itself preclude high susceptibility. It suggests that considerably greater age may do so and indicates, as have other experiments involving younger animals, that individuals differ decidedly in both initial susceptibility and ability to acquire resistance to lungworm infection.—THOMAS B. WEBER, *Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland,* (resigned July 1957).

THE DISTRIBUTION OF *SYPHACIA OBVELATA* IN THE INTESTINE OF MICE DURING THEIR MIGRATORY PERIOD

Biological studies on *S. obvelata* have been hampered by the inability of investigators to secure large numbers of infective eggs for experimental infection of laboratory animals. It has been shown repeatedly that eggs macerated from female *Syphacia* recovered from the cecum of mice failed to develop *in vitro* (Philpot, 1924, J. Helminthol. **2:** 239–252) (Prince, 1950, Science **111:** 66–67) (Chan, 1952, Amer. J. Hyg. **56:** 14–21) Gravid *S. obvelata* migrate out of their hosts for egg-deposition and egg-dissemination at the end of their life cycle. We have, therefore, been employing migrating worms from the colon of infected mice as the source of infective eggs. Recently, a method for recovering large numbers of these worms from mice was made possible by ascertaining the distribution of *S. obvelata* in the intestine of mice during their migratory period. This enabled us to sacrifice experimental animals during the period when maximal migration of worms to the colon may be expected. The eggs of migrating worms become infective after an incubation period of 20 hours at room temperature or 3 to 5 hours at 37° C. They may also be stored at 1–3° C for future use and remain infective for 10–14 days (Chan 1956, J. Parasit. **42:** (Sect. II) 18).

TABLE I. *The distribution of* Syphacia obvelata *in the intestine of mice during their migratory period.*

No. of days after infection	Mouse No.	WORM BURDEN			
		Cecum	Colon	Total	Swab
10 days	1	99	0	99	neg.
	2	106	0	106	neg.
	3	97	0	97	neg.
	4	72	0	72	neg.
	5	159	0	159	neg.
11 days	1	50	0	50	pos.
	2	42	0	42	pos.
	3	81	1	81	neg.
	4	102	0	102	pos.
	5	51	0	51	pos.
	6	78	0	78	pos.
12 days	1	23	74	97	pos.
	2	105	80	185	pos.
	3	30	4	34	pos.
	4	114	20	134	pos.
	5	79	85	164	pos.
	6	59	5	64	pos.
	7	38	7	46	pos.
	8	52	17	79	pos.
13 days	1	52	1	53	
	2	8	0	8	
	3	18	0	18	
	4	4	0	4	
14 days	1	7	0	7	
	2	4	0	4	
	3	6	0	6	
	4	2	0	2	
15 days	1	4	4	8	
	2	12	1	13	
	3	50	1	51	
16 days	1	1	0	1	
	2	1	0	1	
	3	0	0	0	

Thirty-four male mice, 3 to 4 weeks old and weighing between 16 to 20 grams obtained from our nematode-free mouse colony were placed in contaminated pans with heavily infected *Syphacia* "source" mice for 24 hours. From 3 to 8 animals were then sacrificed at 24-hour intervals for 6 days beginning 10 days after infection. The total worm-burden of each mouse was determined and their location in the host's intestine noted. A cellophane anal swab was taken and examined from each animal of the groups killed 10 to 12 days after infection before autopsy.

Table I summarizes the results of this study. The maximal number of gravid worms appeared to be present in the colon in the 24-hour period beginning 12 days after infection. The worm-burden of the 8 mice killed on the 12th day of the infection averaged 100; of these, 64% were in the cecum and 36% in the colon. The majority of the worms recovered from the colon shed their eggs instantly on exposure to air or in saline solution, each depositing an average of 300 eggs. The swabs applied to the perianal region of mice were positive for *Syphacia* eggs as early as 11 days after infection indicating the migration of the worms to the colon and from the host at that time.

Mice infected by the direct feeding of infective eggs of *S. obvelata* through a stomach tube instead of by association with infected mice yielded similar results. Migration of the gravid worms occurred predominantly 12 days after infection. Either of these methods may be used to infect mice for securing infective eggs of *S. obvelata*.—K. F. CHAN AND SYDELL KOPILOF, *School of Public Health and Administrative Medicine, Columbia University.*

INEFFICACY OF RABBITS FOR TESTING ANTHELMINTICS TO BE USED AGAINST GASTRO-INTESTINAL NEMATODES OF RUMINANTS

The report of Bull (1953, N. Zeal. J. Sci. Technol. **34**: 341–372) of natural infection with *Trichostrongylus axei* in a wild rabbit in New Zealand, and the demonstration by Drudge *et al* (1955, J. Parasitol. **41**: 505–511) that rabbits are susceptible to experimental infection with this parasite, suggest the possible use of these relatively inexpensive animals for work on some aspects of gastro-intestinal nematodiasis of ruminants. The study reported here was undertaken to determine whether rabbits might be used satisfactorily for testing compounds for anthelmintic activity against *T. axei* and, therefore, perhaps other gastro-intestinal parasites of ruminants.

Rabbits (5 to 8 lbs) were experimentally infected with *T. axei* by administering 6,000 to 9,000 larvae into the throat as described by Drudge *et al*. Six to 9 weeks later the anthelmintics (phenothiazine, piperazine and "Cu-nic"), in liquid medium, were administered into the esophagus in a similar manner, the rabbits having been lightly anesthetized with ether.

Phenothiazine in doses of 200 and 110 mg/kg respectively removed approximately 100% and 90% of *T. axei* from calves (Porter, 1941, Proc. Helm. Soc. Wash. **8**: 38–41). In 8 rabbits given doses of 660 to 2,000 mg/kg of phenothiazine, there was only 3% change in the average worm egg-per-gram counts of feces on the 3rd to 6th or 8th day after treatment as compared with the average counts for 3 days before treatment, although phenothiazine usually stops or retards egg production temporarily even when it does not expel the worms.

Piperazine citrate was shown by Swanson *et al* (1957, J. Am. Vet. Med. Assoc. **130**: 252–254) to have removed 16% of *T. axei* from calves when given at a rate of 313 mg/kg, and to have been an effective anthelmintic against *O. radiatum*, *O. ostertagi* and *Cooperia* spp. at a dose rate of 155 mg/kg. Three rabbits given 350, 500 and 1775 mg/kg of anhydrous piperazine, respectively, failed to pass any *T. axei* for 4 days following treatment, although at necropsy on the 5th day, using the digestion method of Herlich (1956, Proc. Helm. Soc. Wash. **23**: 102–103), 850, 1160 and 480 worms were found in the 3 rabbits, respectively.

Cu-nic (1.75%/$CuSO_4$ and 0.8% Black Leaf 40) has long been recognized as a valuable anthelmintic in sheep and cattle, particularly against *Haemonchus*, although its efficacy is somewhat erratic, apparently due to occasional entrance into the rumen instead of the abomasum. This drug was given to 3 rabbits in doses of 6, 8 and 10 ml, respectively, corresponding to about 60 to 100 ml in sheep (usual dose 25 to 60 gm/kg). On 2 days following treatment the worms recovered in the feces were, respectively, 100, 100 and 10; no worms were found on the 3rd day. The unexpelled worms found at necropsy were 380, 260 and 630. It is evident that even in rabbits, where the drug must always reach the worms directly, the results are quite erratic and of a low order of efficacy.

From this it is evident that rabbits are not satisfactory animals for tests of anthelmintics to be used against *T. axei* in ruminants.

The author expresses appreciation to Dr. Asa C. Chandler for aid in writing of manuscript.
—GEORGE E. CAUTHEN, *Texas Agricultural Experiment Station, Substation No. 3, Angleton, Texas.* (Technical Article No. 2674)

MASS CULTIVATION OF *AUSTRALORBIS GLABRATUS*, INTERMEDIATE HOST OF *SCHISTOSOMA MANSONI* IN PUERTO RICO

Using a unit consisting of six 10-gallon glass aquaria and a carefully routinized schedule of feeding and water change, it has been possible to obtain a regular weekly yield of 500 to 800 young but sexually mature *Australorbis glabratus* snails per week. Over 15,000 snails have been produced by this method in 6 months from tanks occupying only 9½ square feet of table space.

Each aquarium is fitted with a hammock of fine-mesh nylon net material which extends down into the water to a depth of 3 or 4 inches. The hammock serves to hold the snails during their development and provides a simple means of transferring the colony *en masse* when the water needs changing. One aquarium contains 15 to 20 large snails which serve as egg producers for the colony. They lay their eggs on pieces of freshly cut water lily leaf which are provided for egg laying 3 days out of each week. Five hundred to 800 of these eggs (10 to 20 egg masses) are transferred weekly with as little attached leaf as possible from this aquarium to 1 of the other 5 aquaria. The 5 aquaria are stocked with eggs in rotation, and snails which have become 5 weeks old (usually 5 to 10 mm in diameter) are used or discarded to make room for a new group.

Water from a biologically balanced pond is used exclusively in all aquaria. To obtain the maximum rate of growth, the water in each of the 5 aquaria containing young *Australorbis* is changed every 11 or 12 days during the 5-week growth period. With this exception, no other water changes are necessary as long as the snails continue to eat well at the surface and on the bottom of the nylon hammock. Clouding of the water can be disregarded unless the snails appear to congregate along the nylon at the surface, or attempt to leave the water. No more than 800 snails should be kept in 1 aquarium, even while the snails are small. Crowding causes the water to foul rapidly, thereby requiring many changes and excessive time and labor. Several small fish, placed in or beneath the nylon hammock, will halt mosquito production if this becomes a problem.

Two types of food are provided to both young and mature snails. Small pieces of tender head or leaf lettuce are fed in excess so that several pieces are always present in each hammock. In addition, a dry mixture of food is prepared as follows:

50 parts Purina Dog Chow, Kibbled Meal (ground to a dry powder in an electric blender and sifted through 16-mesh screen)
50 parts dry silt (screened)
1 part Calcium Carbonate, CP.

The powdered mixture is sprinkled on the surface of the water once or twice each day, but the snails are never given more than they can consume in 1 day. Aquaria containing newly hatched snails should receive only a few grains each day. Aquaria containing older snails require more of the preparation daily.

Mortality among young snails is usually insignificantly low. If excessive deaths occur, it usually indicates contamination of the water or of laboratory glassware which has contacted the water.

Australorbis produced by this method are being used at present as the test animal in a possible method of assaying the *S. mansoni* egg burden of raw sewage and sewage treatment plant effluent. The writer believes that the culturing procedure will prove useful to individual research workers who have limited space and time as well as to groups that need many thousands of snails each week for molluscicide research, cercarial antigen preparation, or other purposes.—WILLIAM B. ROWAN, *U. S. Department of Health, Education, and Welfare, Public Health Service, Communicable Disease Center, Puerto Rico Field Station, San Juan, Puerto Rico.*

Seligson AUTOMATIC PIPETTE*

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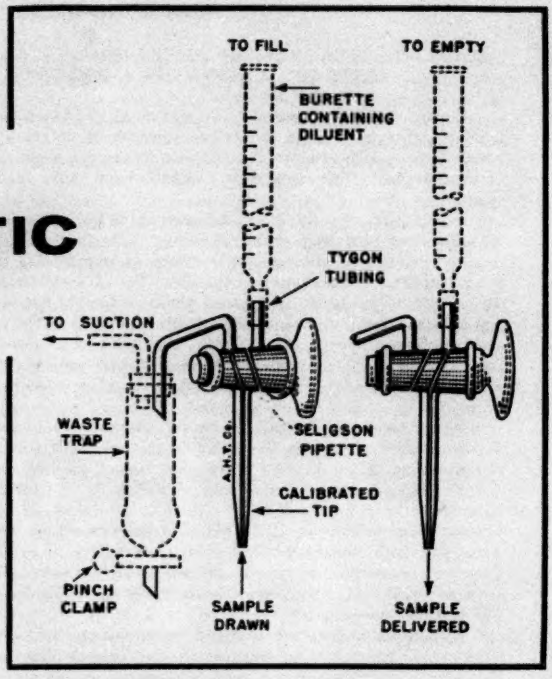

. . . eliminates individual sampling pipettes and repeated filling of dilution pipettes

A fixed volume pipette permanently attached to a double bore stopcock, which permits rapid filling without adjustment to a reference mark, and complete washout delivery of the contained sample by a measured amount of a second liquid from a burette, pipette, or arbitrarily calibrated tube connected to upper arm. Eliminates need for individual sampling pipettes, and repeated filling of dilution pipettes, realizing a highly significant economy of time and glassware, and assuring reproducible sampling, delivery, and dilution which are especially important in routine clinical chemistry. Applicable, however, to any type analysis in which a standard volume is diluted or reacted directly with a measured volume of a second liquid.

In use, the pipette is attached to the dispensing burette by means of the upright arm of the stopcock, and to a suction device by means of the bent tube at the left. A waste trap is inserted into the line prior to the suction connection.

Samples are taken by setting stopcock to connect tip with suction line. A small excess is drawn over into the bent tube to serve as an initial rinse for the pipette wall. The sample is delivered by reversal of the stopcock plug to allow solution in the burette to expel the sample through the tip, and to rinse it clean. Following delivery of the required amount of reagent from the burette, the tip, filled with diluent which keeps it clean, is ready for immediate intake of the next sample.

The pipette, devised by Dr. David Seligson of the Graduate Hospital of the University of Pennsylvania, has been applied to more than twenty-five clinical chemistry procedures as described in *American Journal of Clinical Pathology, Vol. 28, No. 2 (August, 1957) pp. 200-207*, and *Technical Bulletin of the Registry of Medical Technologists, Vol. 27, No. 7 (July, 1957) pp. 152-159.*

The pipettes are designed for attachment to standard burettes or to similar dispensing vessels. Connection is best made with Tygon tubing, which makes a transparent, flexible joint.

8209-B. Pipettes, Seligson, as above described.

To contain, ml.....	0.05	0.1	0.2	0.5	1.0
Tolerance, ml (±)...	0.005	0.005	0.01	0.01	0.05
Tip length, mm.....	65	55	65	160	140
Each.....	11.00	11.00	11.00	11.00	11.00

*Patent applied for.

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